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14858 P

Temperature Reactions in Dogs to Inoculation with Ferret Passage Distemper Virus.

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Serial passage through ferrets of a distemper virus virulent for dogs, foxes, and ferrets results in a modification[1] of the virus tending toward higher virulence for the ferret and lower virulence for foxes and dogs. Within this zoologic modification related to animal species, additional modifications of the virus may be accomplished by the consistent selection of a single tissue, such as skin, brain, or spleen, for use as transmission virus. The histologic modifications effected by selection of tissue seem to produce viruses that have distinct abilities to invade to a greater or lesser extent the various types of susceptible cells. The study of temperatures induced by these viruses was stimulated by the observation that, upon injection into dogs, smaller doses of the ferret-passage virus appeared to excite more marked clinical reactions than did larger doses. In utilizing various doses of ferret-passage virus furnished by us, Stader and Slaughenhaupt[2] noted milder reactions in dogs from 15 mg of virus than from 2.5 mg. Similar observations were made by Schlotthauer.[3]

Dogs from 8 to 12 weeks old were selected for these experiments but were chosen without special consideration as to their weight. Although they varied considerably in the latter respect, the variation in weight was much smaller proportionally than the variation in the doses of virus used. The differences in weight were minimized by the division of litters to put as nearly as possible an equal number of littermates in each group. The dogs were housed in small kennels in outdoor yards. The temperatures were taken during the afternoon, usually at 3:30.

Two ferret-passage viruses of 54 and 63 serial transfers, respectively, were studied with regard to the temperature reactions they induced in dogs when injected subcutaneously in a small dose and in a large dose. The virus was prepared by subcutaneous injection of spleen tissue and the subsequent collection of

* Aided by funds and facilities furnished by Fromm Laboratories, Grafton, Wisconsin.

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2 Stader, Otto, and Slaughenhaupt, R. R., *No. Am. Vet.*, 1942, **23**, 782.

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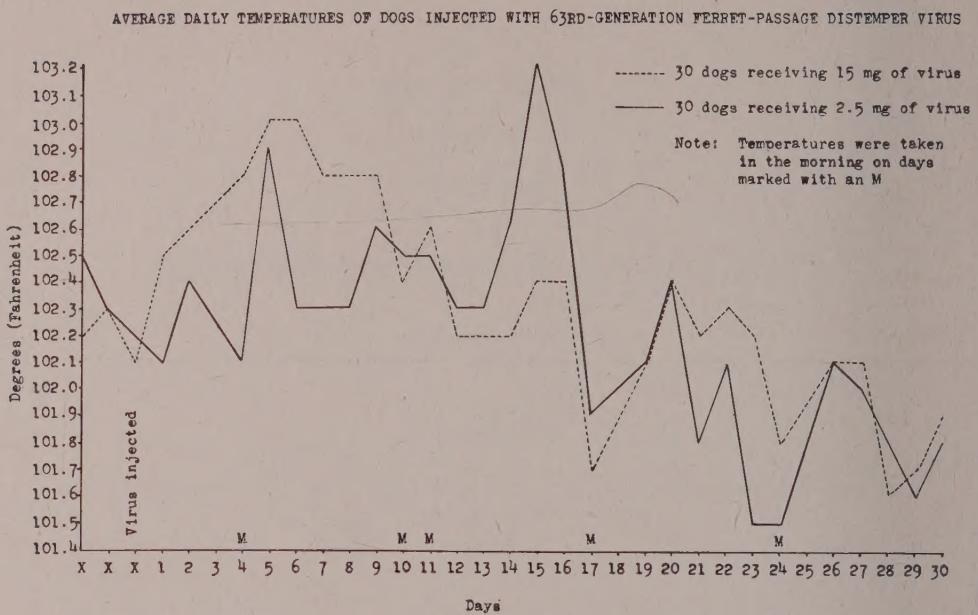

FIG. 1.

spleen tissue for virus. The 54th-generation virus was injected in doses of 2.5 and 15 mg into 25 dogs each. The 63rd-generation virus was injected in doses of the same sizes into 30 dogs each. The results obtained from inoculation of the 110 dogs included in these experiments would seem to demonstrate that, as determined by average daily temperatures, ferret-passage distemper virus of 63 generations shows somewhat of an inverse relationship between dose and degree of temperature reaction.

The temperature reactions of dogs with distemper were first studied by Laidlaw and Dunkin,[4] who described the biphasic temperature curve now considered typical of distemper in dogs. Usually, an initial rise in temperature occurs about the 6th day. The temperature then declines and a second rise occurs about the 14th day, following which a variable fever is maintained during the course of the disease.

In our studies, we found that both the small dose and the large dose of 54th-generation ferret-passage distemper virus produced a mild infection with a biphasic temperature reaction such as that typical of a natural distemper infection. No marked differences were noted in the average daily temperatures produced in the dogs by the 2 different doses. However, in the dogs injected with 63rd-generation virus, the dose of 2.5 mg produced the prolonged biphasic temperature reaction while the dose of 15 mg produced only a single rise in temperature, which was followed by a gradual decline to approximately normal temperatures by the 16th day. The average daily temperatures of the 2 groups of dogs injected with 63rd-generation virus are shown in Fig. 1.

An analysis of the individual temperature records reveals that of the 30 dogs receiving the small dose, 2.5 mg, of 63rd-generation virus, 8 did not show a significant temperature reaction and did not appear to have developed an infection. A graph of the average daily temperatures of the remaining 22 dogs, which showed definite fever, does not differ materially from the curve in Fig. 1, in which the temperatures of all 30 dogs in the group were utilized. Analysis of the individual temperature records of the dogs receiving 15 mg of 63rd-generation virus shows that 23 dogs developed high initial temperatures which

[4] Laidlaw, P. P., and Dunkin, G. W., *Vet. J.*, 1928, **84**, 600.

gradually declined. A curve of the average daily temperatures of these dogs does not differ significantly from the curve of all 30 dogs in the group as shown in Fig. 1. The other 7 dogs of this group had no significant initial rise in temperature but developed a late elevation about the 18th to 22nd day. In these 7 dogs the larger dose of 15 mg produced temperature reactions somewhat similar to those produced by the smaller dose.

Previous reports[5] have recorded the low pathogenicity of ferret-passage virus. In the present experiments, 1 dog of the 50 injected with 54th-generation virus was destroyed on the 14th day, and 1 of the 60 injected with 63rd-generation virus was destroyed on the 15th day when they developed prolapse of the rectum. One dog injected with 63rd-generation virus died in convulsions on the 5th day, but the fatality did not seem to be related to the inoculation.

Conclusions. Ferret-passage distemper virus of 54 serial transfers, upon injection into young dogs, induced a mild infection with a biphasic temperature reaction similar to that observed in natural distemper infections. No difference in temperature reactions was observed in groups of dogs injected with 2.5 mg and with 15 mg of the ferret-passage virus. Similar mild reactions were observed following the injection of virus of 63 ferret passages. The dose of 2.5 mg produced temperature reactions which were biphasic in character. The 15-mg dose of this virus tended to produce an exaggerated initial fever which gradually declined without the production of a second rise in temperature.

[5] Green, Robert G., *Am. J. Hyg.*, 1945, **41**, 7.

14859

The Influence of Muscle Pain on Spinal Reflexes.*

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Introduction. In a previous investigation[1] the effect of intramuscular injection of NaCl solution on the response of the motor cortex to electrical stimulation has been studied. It was shown that the effect of cortical stimulation was altered quantitatively and qualitatively under these conditions. Since evidence from our own experiments based on reflex effects, particularly on the pupil, as well as from studies of Lewis,[2] on man, suggested that injection of hypertonic NaCl solution caused "muscle pain," the experiments were interpreted to mean that "muscle pain" altered the effect of cortical stimulation of the motor area on the organism. The physiological mechanism involved in these reactions has not yet been determined. As a first step in this direction experiments are reported in this paper in which the influence of "muscle pain" (*i.e.*, of stimuli known to cause muscle pain in man and to induce pupillary dilatation and vocalization in anesthetized animals) on spinal reflexes was investigated.

Method. The experiments were performed on eleven cats and one macaque. .45 cc dial-urethane (Ciba)† /kilo was injected intraperitoneally. The knee jerk was elicited every two seconds by means of a device similar to that described by Johnson.[3] The flexor reflex (tibialis ant.) was studied by means of condenser discharges applied at intervals of 2.5 seconds. In some of the animals studied the spinal cord was transected at the first lumbar level. As stimuli which result in muscle pain

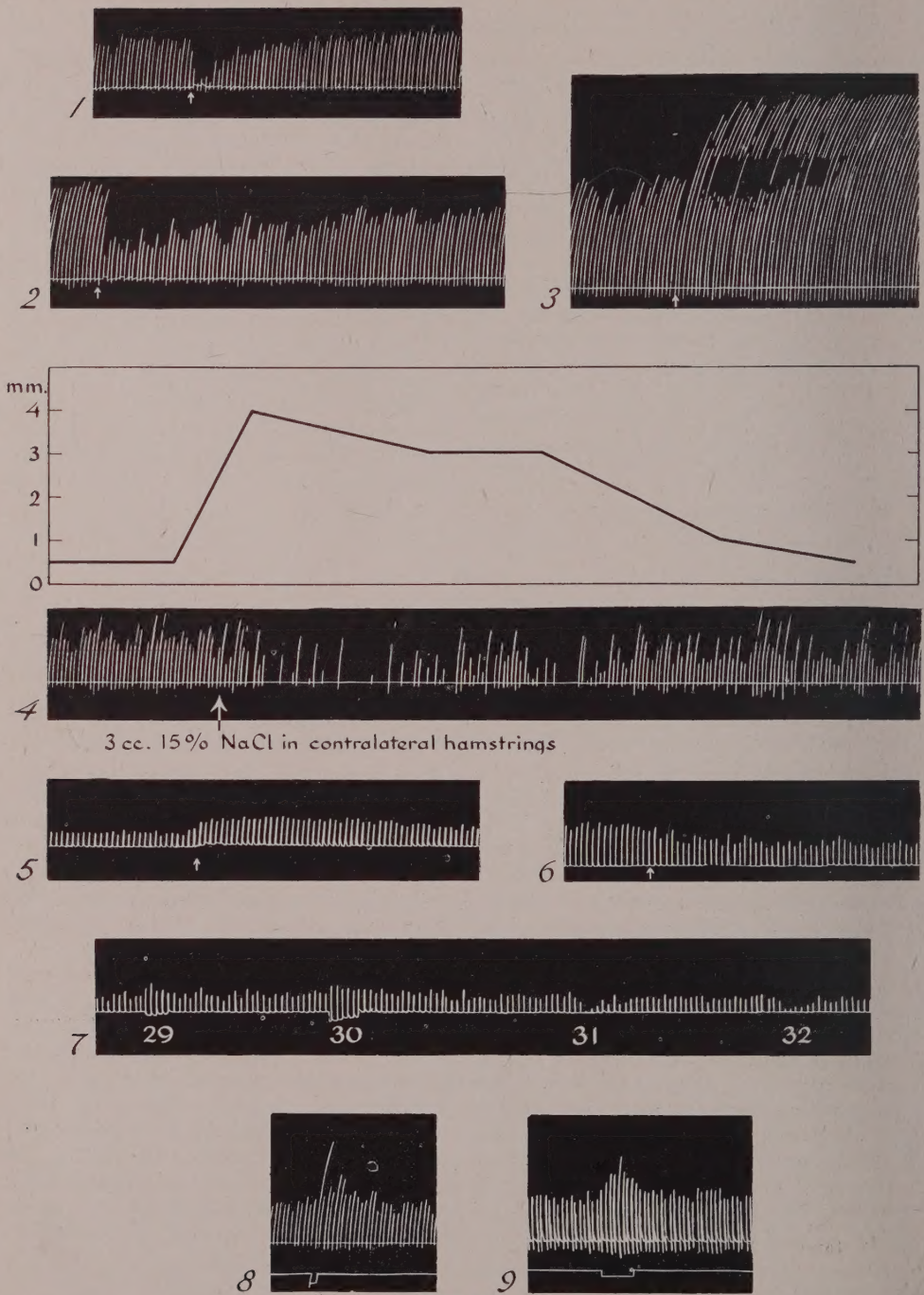
* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Gellhorn, E., and Thompson, L., *Am. J. Physiol.*, 1944, **142**, 231.

[2] Lewis, T., *Pain*, New York, 1942.

† Ciba Pharmaceutical Products, Inc., was kind enough to donate this drug.

[3] Johnson, C. A., *Am. J. Physiol.*, 1927, **82**, 75.



FIGS. 1-4.

Knee jerk elicited every two seconds in cats anesthetized with .45 cc dial-urethane. At the arrow 15% NaCl was injected intramuscularly as follows:

Fig. 1, 2 cc into ipsilateral hamstrings; Fig. 2, 1 cc ipsilateral hamstrings; Fig. 3, 2 cc contralateral gastrocnemius; Fig. 4, 3 cc contralateral hamstrings. The graph above the knee jerk record of Fig. 4 shows the change in pupillary diameter resulting from the injection of hypertonic NaCl solution.

FIGS. 5, 6, 7, AND 9.

Flexor reflex (tibialis ant.) of the cat anesthetized with dial-urethane was elicited by condenser discharges applied at intervals of 2.5 sec. to the central end of the sciatic ipsilateral (intensity 5-6 volts). At the arrow 2 cc 15% NaCl were injected intramuscularly into the ipsilateral hamstring (Fig. 5) and 3 cc of this solution into the contralateral hamstrings (Fig. 6). Fig. 7 shows the effect of faradization of muscles on the flexor reflex. Nos. 29 and 30 ipsilateral faradization of the quadriceps at 11 and 9 cm c.d. respectively. Nos. 31 and 32 contralateral faradization of the gastrocnemius at 9 cm c.d. Fig. 9: Effect of faradization (6.5 cm c.d.) of the ipsilateral quadriceps on the flexor reflex.

FIG. 8.

Macaque .55 cc dial-urethane/kilo i.p. Effect of faradization (6 cm c.d) of the contralateral quadriceps on the knee jerk.

in man we used injection of 15% NaCl solution or faradization of ipsi- and contralateral muscles.

Results. The experiments showed that the injection of 15% NaCl as well as the faradization of muscles influence the knee jerk and the flexor reflex of cat and monkey. Fig. 1 and 2 demonstrate the effect of injection of a hypertonic NaCl solution into the ipsilateral muscles on the knee jerk. This reflex is regularly depressed under these conditions, but the effect may vary greatly in intensity and duration as shown in these figures. It may, however, be emphasized that apparently due to the slowness of diffusion processes the effect resulting from the injection of hypertonic solutions may persist for several minutes. It is reversible and accompanied by dilatation of the pupil and, in some instances, by vocalization. When NaCl was injected into muscles of the contralateral side the results were not uniform. In seven experiments an increase in the knee jerk was observed whereas in three experiments the knee jerk was actually diminished. Examples for the two types of results are shown in Fig. 3 and 4. Contralateral injection of NaCl caused a distinct increase in the knee jerk in Fig. 3, whereas in Fig. 4 it was accompanied by a prolonged inhibition of this reflex.

As was mentioned in the introduction, faradization of muscles likewise causes muscle pain in the human. Therefore this stimulus was used in order to study its effects on the knee jerk. It was found that ipsilateral faradization inhibited the knee jerk whereas contralateral muscle faradization increased the effect. These effects were temporary and lasted only as long as the stimulus was applied. Neither the injection of NaCl nor the faradization of the muscle caused in themselves, a reflex on the recorded muscle.

In a second group of experiments the effect of these stimuli on the flexor reflex was studied. Fig. 5 and 6 illustrate the action of ipsi- and contralateral injection of hypertonic NaCl solution on the flexor reflex. Whereas ipsilateral "muscle pain" increases the flexor reflex, contralateral "muscle pain" decreases it. This statement applies for the "muscle pain" resulting from the injection of hypertonic NaCl solution (Fig. 5 and 6) as well as for the faradization of muscles (Fig. 7). It is apparent from the description of these experiments that in general the extensor reflex (knee jerk) and the flexor reflex react in opposite manners to the same type of stimulus. This is further illustrated by Fig. 8 and 9 in which it is shown that contralateral faradization increases the knee jerk in a similar manner as ipsilateral faradization effects the flexor reflex. The converse statement is likewise true but an illustration has been omitted.

Discussion. Since the effects described in the preceding paragraphs were obtained in animals with the spinal cord transected at the lumbar level as well as in animals with the spinal cord intact, it may be inferred that they represent effects on the spinal centers. It seems therefore justified to conclude that osmotic or faradic stimulation of pain fibers in the muscle leads to an alteration of the excitation of spinal centers which is demonstrated by marked changes in spinal reflexes in response to a standard stimulus. Although the stimuli used in these experiments led, in

many instances, to profound alterations of the reflexes for several minutes, they did not cause any reflexes by themselves. However, they acted, in general, as was to be expected if Sherrington's rules could be applied to nociceptive stimuli although the latter did not evoke any reflexes. Since ipsilateral nociceptive stimuli elicit flexor- and inhibit extensor-reflexes whereas contralateral stimuli act in opposite manners ipsilateral nociceptive stimuli may be expected to increase flexor- and decrease extensor- (knee jerk) reflexes, and opposite effects should be produced by contralateral nociceptive stimuli. Our results show that this is, in general, the case. However, it is worthy of mention that, in several instances, contralateral stimulation of pain fibers by means of hypertonic NaCl solution markedly diminished the flexor reflex. The prolonged and sometimes considerable increase in pupillary diameter which accompanied this inhibitory effect (*cf.* Fig. 4) makes one suspect that this deviation from the rule occurred particularly when excessive pain was induced.

It was shown earlier (Gellhorn and Thompson, 1944) that the effects of electrical stimulation of the motor cortex were greatly increased by means of painful stimuli applied to the muscles of the limbs. In contradistinction to the influence of "muscle pain" on spinal centers described in this paper it was found that its action on the effect of cortical stimulation was independent of the "laterality" of the pain stimulus. Thus cortical stimulation of the left motor area lead to an intensification of the movement of the right foreleg after injection of hypertonic NaCl solution in either the right or the left hindleg. Apparently the alteration of the effects of cortical stimulation as a result of "muscle pain" is not simply the result of the action of these painful stimuli on spinal centers, although these centers are likewise profoundly influenced.

Summary. Stimuli such as injection of hypertonic NaCl solution into striated muscle or faradization of muscles which cause "muscle pain" in man, and pupillary dilatation and vocalization in the anesthetized animal modify spinal reflexes in the latter. It is shown that in general ipsilateral "painful" stimuli increase the response of a flexor reflex and diminish the response of the knee jerk to a standard stimulus. On the other hand, contralateral "pain" stimulation causes an increase in the knee jerk and a decrease in the flexor reflex. If excessive stimuli are used the knee jerk may be inhibited not only from the ipsi- but also from the contralateral side. The fact that "muscle pain" greatly enhances the effect of electrical stimulation of the motor cortex is not due to the alteration of spinal centers but suggests the involvement of supraspinal mechanisms.

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Effect of Tobacco Virus, Some Decomposition Products of Nucleoproteins, and Related Compounds on Acetylcholine Synthesis.*

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In the presence of serum,[1,2] spinal fluid,[3] and thymus[4] from patients with myasthenia gravis less acetylcholine was synthesized *in vitro* than in the presence of similar tissues

*This investigation was aided by a grant from the John and Mary R. Markle Foundation.

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[3] Torda, C., and Wolff, H. G., *Science*, 1944, **100**, 200.

[4] Trethewie, E. R., and Wright, R. D., *Australian and New Zealand J. Surg.*, 1944, **13**, 244.

from subjects without myasthenia gravis. Patients with myasthenia gravis may have an enlarged thymus and an increase of lymphoid tissue, tissues known to be important for the metabolism of nucleoproteins in the body,[5,6,7] and these patients are sometimes benefited by removal or irradiation of the thymus. Some extracts of the thymus decrease the synthesis of acetylcholine.[8] Irradiation of tissues or degenerative changes of the thymus lead to disturbances in the metabolism of nucleoproteins.[9,10]

On the other hand, some of the decomposition products and related substances of nucleoproteins may sensitize the effector cells to acetylcholine,[11] may cause vasodilatation,[12-22] and it is possible that adenosinetriphosphate supplies the energy required for the synthesis of acetylcholine.[23] In the following it was ascertained whether tobacco virus, some of the decomposition products of nucleoproteins, and some related compounds modify the synthesis of acetylcholine.

Method. The synthesis of acetylcholine was studied by the method of Quastel, Tennenbaum, and Wheatley[24] with minor modifications.[2] Varying amounts of the substances were added to mixtures containing 100 mg minced fresh frog brain, 3 mg physostigmine salicylate, 4.8 mg glucose, and 3 cc Ringer's solution. The pH of the mixtures was adjusted to 7.4. Identical mixtures without the substances served as controls. The mixtures were shaken and incubated aerobically for 4 hours at 37°C. After incubation the amounts of free and total acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog. The amount of acetylcholine synthesized was calculated by subtracting from the acetylcholine content of the incubated mixtures the acetylcholine content of identical non-incubated mixtures. By adding the substances in varying concentrations to incubated control mixtures after incubation it was ascertained whether the substances modified the sensitivity of the rectus abdominis muscle to the acetylcholine content of the mixtures during the 2 minutes of immersion for the biological assay. If so the changes were taken in account by the calculation.

Results. The amounts of acetylcholine synthesized in the presence of the substances used are given in Table I. Within the range of concentrations used tobacco virus and the methylxanthines increased the amount of acetylcholine synthesized, whereas many of the decomposition products of nucleoproteins decreased the synthesis of acetylcholine. These results suggest the possibility that some of the decomposition products of nucleoproteins occurring in the body exert an unfavorable effect on the synthesis of acetylcholine (*e. g.* muscle inosinic acid, an usual constituent of the mammalian striated muscle, a product

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TABLE I.
Effect of the Substances on the Synthesis of Acetylcholine.

		Amt of acetylcholine synthesized in % of control*									
		Free acetylcholine					Total acetylcholine				
		Amts of substances in mg added to 100 mg frog brain:									
Substance	No. of exp.	10	3	0.3	0.03	0.003	10	3	0.3	0.03	0.003
Tobacco virus	4			135	124				147	127	
Nucleic acid (yeast)	10		30	85	87			26	79	83	
Adenylic acid	10		127	119	108	91		122	115	110	100
Inosinic acid (muscle)	8			68	75	80			70	72	76
Adenosine	10		73	90	117			77	84	103	
Adenine sulfate	10	33	57	68	90	95	35	45	63	86	99
" acetate	8		40	65	80	86		39	60	80	86
Guanine	10		27	75	103			48	77	96	
Xanthine	10	85	82	84	81		72	82	90	92	
Uracil	10	80	102	97	106		85	105	100	107	
Thiouracil	10	76	75	81	104		75	83	87	100	
Uric acid	12	53	58	74	74		43	55	66	88	
Caffeine	10		164	150	130	101		170	142	126	93
Theobromine	10		170	148	135	110		178	157	130	112
Theofilline	10		165	150	130	105		177	156	120	100
Pyridine	12	43	77	83	103		66	83	81	90	
2-methyl-5-ethoxy-methyl-6-amino pyrimidine	8		60	68	75	82		61	66	78	84
4-methyl-5-β-mydroxyethyl thiazole	6		30	48	55	81		20	43	51	80
Pyrol	10	57	75	85	94	105		77	83	94	100
Piperidine	8		11	156	107	95		42	155	119	105
Alloxan	10		45	57	77	90		40	59	80	88
Methylguanidine	10		60	96	96	117		58	103	100	118
Urea	10	96	139	112	115		101	133	110	110	
Thiourea	10	96	97	99	107		106	104	105	116	

* The S.E. of the mean for each value was less than $\pm 5\%$. The amounts of acetylcholine synthesized in μg per 100 mg frog brain, followed by the S.E. of the mean were: Free acetylcholine, 0.70 ± 0.023; total acetylcholine, 1.50 ± 0.044.

formed also during muscle activity by deamination of muscle adenylic acid).

Summary and Conclusions. 1. The effect of tobacco virus, some of the decomposition products of nucleoproteins, and some related substances on the synthesis of acetylcholine was investigated. 2. Tobacco virus, methylxanthines, adenylic acid, and urea increased the synthesis of acetylcholine. 3. The synthesis was not modified by thiourea. 4. Piperidine increased the synthesis in lower concentrations and decreased it in higher ones. 5. Methylguanidine and uracil did not modify the synthesis in low concentrations and decreased it in higher ones. 6. The synthesis of acetylcholine was decreased in the presence of nucleic acid, inosinic acid, adenine, guanine, xanthine, adenosine, thiouracil, uric acid, pyridine, 2-methyl-5-ethoxymethyl-6-amino pyrimidine, 4-methyl-5-β-hydroxyethyl thiazole, pyrol, and alloxan.

The authors wish to express their gratitude to Merck & Co., Inc., for the generous supply of 2-methyl-5-ethoxymethyl-6-amino pyrimidine and 4-methyl-5-β-hydroxyethyl thiazole, and to Dr. Vincent DuVigneaud for the tobacco virus and adenosine and to the Rockefeller Institute, N. Y. for the muscle inosinic acid.

14861 P

Effect of Cell Growth Activating Tissue Extracts, Parenterally Applied, on Experimental Skin Wounds.*

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In the preceding paper[1,2] we showed that the local application to experimental skin wounds of cell growth promoting preparations, obtained from adult as well as embryonic tissues, does not result in an acceleration of the healing process in the treated wounds, as compared with control wounds in the same animal. It is necessary to stress, however, that on closed examination of the data presented[2] it becomes evident that both the treated and control wounds in the same experimental animal, compared with wounds of the same size and location in untreated animals close more rapidly. This suggests that the results of our experiments employing local treatment of experimental skin wounds in rats were only apparently negative. It seems likely that the locally applied cell growth promoting extracts do exert an accelerating action on the rate at which the wounds heal. This acceleration, however, could not be perceived from a comparison with control wounds in the same animal, since the healing process in the latter, too, was hastened. Thus, the possibility must be envisaged that the effect of cell growth-promoting tissue extracts on wound healing is not local, but of a general nature.

As an approach to this question experiments were undertaken to determine the effect on wound healing of cell growth-promoting adult tissue extracts administered parenterally. The wound-healing process in animals treated in this way was compared with that in untreated control animals.

TABLE I.
Results of Wound Healing Experiments.*

Exp. No.	Closure time in days in rats	
	Treated	Untreated
96	16	23
129	15	22
130	17	16
131	16	22
132	17	20
133	17	19
134	13	20
135	16	23
193	18	21
197	18	24
199	16	20
201	18	21
203	17	18
247	17	22
249	16	19
251	16	28
252	16	24
253	18	27
275	17	22
276	18	20
277	19	22
278	20	21
279	19	21
280	17	21
281	16	21
282	17	21
283	17	21
284	15	21
285	19	25
286	16	22

I. Mean period required for healing in rats
  (a) treated 16.9 days
  (b) untreated 21.6 days
S.E. of mean in rats
  (a) treated ±0.26
  (b) untreated ±0.44
II. Difference between controls and treated 4.7 days
S.E. of difference ±0.51
III. Percentage difference of mean periods, 21.8%

The experiments were performed on white rats, averaging 180 g in weight. In each experiment two animals of equal size and of

* Aided by a grant from the Dazian Foundation for Medical Research, New York.

† Working under the Hadassah Medical Organization Fellowship.

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* The authors wish to thank Dr. Goldhaber and Dr. R. Bachi for their assistance in the statistical treatment of the data presented in this investigation.

the same sex (in most cases littermates) were chosen. One circular wound of 20 mm diameter was produced on the back, the skin being excised down to the superficial fascia.

The technic of producing wounds was that described in the previous paper. The method of treatment consisted in intraperitoneal injections of 1 cc sterile 33% saline extract of adult chicken heart, on every second day. The control animal remained untreated. The wounds of both animals, treated and untreated, were covered with lanolin and sterile gauze, the dressing being changed daily. The experimental and control wounds were measured at intervals of 48 hours. The injections were begun on the day of operation and continued until the wounds were completely healed. The criterion of healing was based on the complete closure of the wounded area.

The results of the present investigations are given in Table I.

These data indicate that in rats treated with intraperitoneal injections of saline extract of adult chicken heart the closure time of the wounds is on the average 21.8% shorter than in the untreated controls. These results are in agreement with earlier experiments of Roulet,[3] Lorin-Epstein,[4] Amorosi,[5] and Sandelin and Björkesten,[6] who observed stimulation of wound healing following injections of extracts of embryonic tissues.

Summary. Rats with experimental skin wounds received intraperitoneal injections of saline extract of adult chicken heart. The course of wound healing in animals treated in this way was compared with the healing process in wounds of equal size in untreated rats. The experiments showed that treatment with cell growth-promoting extracts of adult hearts caused a significant reduction (21.8%) in the mean time required for healing, as compared with the controls.

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14862 P

Liver Changes Associated with a Transplantable Granulosa Cell Carcinoma in Mice.*

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Earlier experiments[1,2] have indicated that irradiation of the entire body of mice results in the development of granulosa cell tumors of the ovary in many of the X-rayed animals. Although many tumors attained a large size, none of the induced ovarian growths of our first series[3] has metastasized to distant organs, and for this reason the neoplastic character of these tumors was questioned.

In the present series, granulosa cell tumors were induced by a single dose of 175 or 350 r. Many of these animals are still alive, and a final analysis of the incidence of ovarian tumors cannot be presented. In one com-

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pleted series of 78 mice receiving 175 r at 4 to 7 weeks of age and living until 10 months of age or longer, ovarian tumors appeared in 49 mice. Many other mice had a proliferative change[4, 5] that precedes the development of tumors.

Four of the induced granulosa cell tumors metastasized to distant organs. Transplantation was attempted with 2 of these growths by grafting pieces of the tumor into the subcutaneous tissue or peritoneal cavity of related mice. Both attempts were successful. A fifth granulosa cell tumor that measured 25 mm. in greatest diameter but had no gross metastases also proved transplantable.

Microscopically, cells of the granulosa cell tumor 1 deviate but little from those of normal granulosa cells, though mitoses are abundant. Occasionally they show the characteristic follicular arrangement resembling normal ovarian follicles. In the neoplastic follicles are noted spaces with pale-stained, homogeneous, precipitated material resembling closely the formation of ovarian follicular fluid. The microscopic appearance of the tumors produced by cellular grafts is indistinguishable from that of the induced tumor. These grafts grow slowly in the subcutaneous tissue, but eventually attain a large size. The tumor was induced by X-rays in an F_1 hybrid mouse, and thus far transfers have been successful in 62 of 172 genetically identical F_1 hybrids receiving subcutaneous grafts of tumor cells, and in only 1 of 20 mice of the parental stocks.

There is a peculiar liver change in most, if not all, of the mice that have carried the transplanted growth for several weeks. This has not been observed in other mice of our colony, either in normals or in those carrying different transmissible tumors or leukemic grafts, but it was noted in some mice with induced granulosa cell tumors of the ovary. A study of 15 livers of mice with transplanted granulosa cell carcinoma 1 shows the following changes:

Branches of the portal vein and liver sinusoids are tremendously dilated, so that the width of the sinusoids is double or triple that of the liver cords (Fig. 1). If the changes are mild, they tend to be focal. When they are advanced, they involve almost the entire organ, and cords of liver cells undergo atrophy. In scattered areas thrombosis occurs, with occlusion of sinusoids leading to necrosis of liver tissue; this may heal with

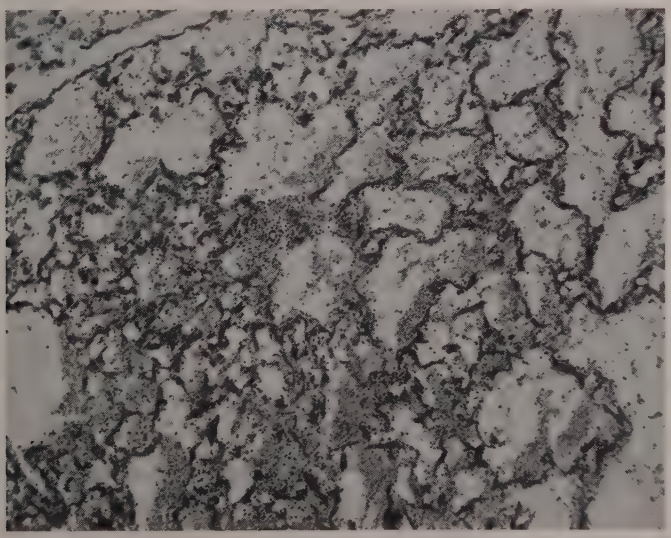

FIG. 1.
Cavernous dilatation of liver sinusoids in a mouse with transplanted granulosa cell carcinoma 1. × 50.

scar formation. The scars are usually focal and disseminated. The massive hyperemia with cavernous dilatation of the sinusoids is usually unaccompanied by any inflammatory reaction; occasionally small lymphoid cells accumulate about cavernous sinusoids. Kupffer cells and large epithelioid cells of undetermined character filled with yellow-brown pigment giving a diffuse yellowish hue to their cytoplasm are present in variable numbers about cavernous sinusoids. There appears to be an increase in the number of Kupffer cells and endothelial cells about many sinusoids. The presence of scattered small hemopoietic foci seen about sinusoids of these mice has also been noted in normal animals at comparable ages.

The character and pathogenesis of this liver damage remain to be determined. The possibility that it is due to a virus carried by and only accidentally associated with granulosa cell carcinoma is unlikely, since in the earlier stage of this liver damage, inflammatory reactions or other changes known to be produced by viruses are absent. It is more probable that it is due to substances derived from the tumor cells. Similar changes have not been described in mice receiving estrogens over a long period of time (*cf.* [6]) or in mice with transplanted granulosa cell carcinoma originating in a non-irradiated mouse.[7] The liver is known to receive and inactivate estrogens, and may undergo swelling with hyperemia during menstruation, and is sometimes profoundly damaged in the course of pregnancy (*cf.* [8]). Thus it can be postulated that a substance of hormonal nature reaches the liver in small quantities under physiological conditions, and that this substance is produced or initiated by granulosa cell carcinoma of the ovary and is discharged in large quantities in animals carrying transmitted granulosa cell growth thereby leading to liver damage. It is also possible that this hypothetical substance is related to, but is not identical with, normal steroid hormones.

Summary. Three granulosa cell tumors of the ovary induced in mice by X-rays have been successfully grafted to related mice, indicating the neoplastic character of this growth.

Associated with the transplantable granulosa cell carcinoma No. 1 is a cavernous dilatation of liver sinusoids, which leads to thrombosis and scar formation. The relation of sex hormones to liver changes is discussed.

6 Gardner, W. U., *Arch. Path.*, 1939, **27**, 138; Allen, E., *Endocrinology*, 1942, **30**, 942.

7 Strong, L. C., Gardner, W. U., and Hill, R. T., *Endocrinology*, 1937, **21**, 268.

8 Eckelt, K., in Halban-Seitz, *Biol. u. Path. d. Weibes*, 1927, **3**, 483.

14863 P

Hypothalamic Facilitation of the Motor Cortex.

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Observations in this laboratory upon a monkey paralyzed from experimental poliomyelitis suggested that emotional states accompanied by hypothalamic activity may elicit cortically induced movements not otherwise operative. It was therefore decided to study the effect of direct stimulation of hypothalamic and adjacent forebrain nuclei upon skeletal muscular responses evoked by electrical stimulation of the cortical motor areas in the experimental animal, in the hope of further clarifying cortico-hypothalamic relations.[1-4]

* Aided by a grant from the National Foundation for Infantile Paralysis.

1 Morison, R. S., Dempsey, E. W., and Morison, B. R., *Am. J. Physiol.*, 1941, **131**, 732.

2 Morison, R. S., Finley, K. H., and Lothrop, G. N., *J. Neurophysiol.*, 1943, **6**, 243.

3 Obrador, S., *J. Neurophysiol.*, 1943, **6**, 81.

4 Kennard, M. A., *J. Neurophysiol.*, 1943, **6**, 405.

Materials and Methods. In cats (dial-urethane) bipolar, steel electrodes (the tips .5 mm apart) were inserted stereotactically (Horsley-Clarke instrument) into the hypothalamic region. Cortical stimulation (bipolar silver electrodes, 2 to 4 mm apart) was effected by use of a Goodwin stimulator, with intensities of from 3 to 5.5 volts, frequencies of 5.5 to 90 impulses per second, and duration of impulse 20 sigma. After the cortex had been stimulated at threshold for 10 seconds, the hypothalamus was stimulated separately for 15 seconds (Harvard inductorium, 3 V, c.d. usually at 8 cm) and the occurrence or absence of sympathetic response (contraction of the nictitating membrane and maximal pupillary dilatation) was observed. Then hypothalamic and cortical stimulation were combined, the former preceding the latter by 5 seconds. Stimuli were spaced at intervals of 2 minutes.

Results. Over 350 experiments were made, on 20 animals, with stimulation of various points in the hypothalamus and adjacent nuclei and with simultaneous stimulation of various cortical areas. In general, the effect of stimulation of a "responsive" area in the basal forebrain nuclei upon simultaneous stimulation of the motor cortex consisted of intensification of the motor response, spread of the response to involve other muscular groups contralateral to or ipsilateral with the motor cortex, shortening of the period of latency of motor response, or—most often—a combination of all three effects. These results were interpreted as being due to facilitation of the motor cortex by stimulation of the hypothalamus.

Exact location of the hypothalamic and adjacent nuclei, discharge of which was found to facilitate the cortical response awaits histological confirmation. However, it is apparent that positivity of a facilitatory effect from hypothalamic stimulation is associated with activation of a nuclear zone productive of sympathetic autonomic effects. 335 experiments were conducted to "map out" the facilitatory area in the basal forebrain. Of the 149 hypothalamic points which were positive for cortical facilitation, stimulation of 120 of these points, or 80%, resulted in retraction of the nictitating membranes and dilatation of the pupils, while the remaining 29 positive facilitatory nuclear points (20%) were negative for concomitant sympathetic response. On the other hand, of the 186 hypothalamic points which did not result in cortical facilitation, 174 points, or 93%, were negative for both types of effect.

That facilitation of the cortex from hypothalamic stimulation does not, however, depend upon associated discharge of the sympathetic nervous system was demonstrated by a series of experiments in which the cervical sympathetic trunks were severed and the adrenals ligated, and by another series in which the medulla oblongata was transected at the point of junction with the cervical spinal cord. In the peripherally and partially sympathectomized animals results were surprising in that far from decreasing the hypothalamic facilitatory effect, facilitation was intensified. Complete elimination of sympathetic effects *per se* by medullary transection did not thus increase cortical facilitation, but neither did it prevent it. It can be said, therefore, that while cortical facilitation by hypothalamic stimulation is obtained almost exclusively from nuclear zones which are sympathetic in their autonomic function, such facilitation is independent of sympathetic activity as such.

The facilitatory effect is likewise not related to ipsilaterality of cortical stimulation, since with the hypothalamic and cortical electrodes contralateral to each other exactly similar results were obtained. Moreover, exploration of the cortex with the central electrodes activating a responsive hypothalamic point revealed that any cortical area productive of motor response was facilitated by hypothalamic stimulation. Another characteristic of this phenomenon is that "frequency differentiation" (production of differing motor responses by stimulation of a given cortical site with varying frequencies of impulse†) is preserved under these conditions.

It appears plausible that activation of the hypothalamus results in discharge of the thalamo-cortical system (dorso-medial nuclei of the thalamus to the cerebral cortex) discovered by Morison, Dempsey, and collab-

† Unpublished observations.

orators.[5,6] Similar efforts to confirm by electrocorticography the gross motor responses described are in process in this laboratory.

The implication of these findings in terms of normally operative physiological activity can, of course, only be conjectured. Observations of the effect of emotional stress on muscle strength, and clinical notes of movements of paralyzed limbs and production of ejaculatory speech in the hemiplegic aphasic[7,8] under conditions of excitement, anger, or joy may have their basis in hypothalamic-cortical facilitation.

[5] Morison, R. S., and Dempsey, E. W., *Am. J. Physiol.*, 1942, **135**, 281.

[6] Dempsey, E. W., and Morison, R. S., *Am. J. Physiol.*, 1942, **135**, 293.

[7] Wilson, S. A. Kinnier, *Neurology*, Baltimore, 1940.

[8] Nielsen, J. M., and FitzGibbon, J. P., *Agnosia, Apraxia, Aphasia*, Los Angeles, 1936.

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Carboxythiazole (2-Sulfanilamido-5-Carboxythiazole) in Blood, Urine and Feces After Oral Administration in Humans.

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Two general types of sulfonamide compounds have been used with some success in the treatment of certain enteric infections and in attempts to reduce the numbers of coliform and other bacteria in the intestinal contents in relation to surgical procedures. One type is represented by sulfaguanidine[1] which is fairly soluble in water, poorly absorbed from the gastro-intestinal tract, and, generally speaking, is about as active as sulfanilamide. The second type is one in which a substitution is made on the *p-amino* group of a more active sulfanilamide derivative, the resulting compound being totally inactive until the added group is released by hydrolysis which occurs within the intestinal tract. Glucose sulfapyridine,[2] succinylsulfathiazole,[3] and phthalylsulfathiazole[4] are examples of such compounds. They, too, are highly soluble, but only a small fraction of the active compound is released in the bowel and low concentrations of the active drug appear in the blood.

Carboxythiazole (2-sulfanilamido-5-carboxythiazole) is a third type of derivative in which a simple substitution is made on the thiazole ring of sulfathiazole.* This results in a compound which, though less active than the original sulfathiazole, is highly soluble and is poorly absorbed. In the present paper are presented the results of studies in human subjects on the occurrence of this drug in the blood, urine, and feces after single and repeated oral doses. The relevant findings in a small number of cases of enteric infections in which this drug was used are also summarized.

Materials and Methods. A single 5 g dose

[1] Marshall, E. K., Jr., *et al.*, *Bull. Johns Hopkins Hosp.*, 1940, **67**, 163.

[2] Taylor, F. H. L., *et al.*, *J. Clin. Invest.*, 1940, **19**, 201; Lowell, F. C., Spring, W. C., Jr., and Finland, M., *ibid.*, 1940, **19**, 215.

[3] Poth, E. J., and Knotts, F. L., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 129.

[4] Mattis, P. A., Benson, W. M., and Koelle, E. S., *J. Pharm. Exp. Therap.*, 1944, **81**, 116.

* This compound was received from the American Cyanamid Co. with the following information: It has a melting point of 215.5°C; water solubility at 37°C and at pH 3.0 of 42.7 mg per 100 cc and a buffer solubility at pH 5.0 of 4920 mg per 100 cc. It is poorly absorbed from the intestinal tract, rapidly excreted from the blood stream, and practically non-toxic in mice, rats, rabbits, and dogs. Experimentally, its antibacterial activity is about equal to that of sulfaguanidine. (Personal communication from Dr. Harold J. White.)

TABLE I.
Recovery of Carboxythiazole from Urine and Feces after a Single Oral Dose of 5 Grams.

Subject A						Subject B					
Urine			Feces			Urine			Feces		
Hr after dose	Free mg	Total mg	Hr after dose	Free mg	Total mg	Hr after dose	Free mg	Total mg	Hr after dose	Free mg	Total mg
0-12	216	230	0-27	1788	1870	0-12	159	236	0-38	0	0
12-24	32	35	27-35	347	354	12-24	31	39	38-46	15	17
24-36	17	26	35-62	347	347	24-36	31	44	46-55	143	148
36-48	17	25	62-120	476	476	36-48	38	58	55-73	127	134
48-72	30	52	120-144	15	16	48-72	40	47	73-84	702	714
72-96	14	19				72-96	56	82	84-107	867	884
96-120	12	28				96-120	71	104	107-149	221	221
0-120	338	415	0-144	2973	3063	0-120	426	610	0-149	2075	2118
Conjugated		19%		3%			30%			2%	
Total amt recovered = 3.478 g or 69.6%								2.728 g or 54.6%			

Blood levels at 3, 6, 12, 27, and 77 hours all 1 mg% (trace) in both subjects.

of carboxythiazole was given by mouth to each of 2 male subjects. One of them (A) was given a mild laxative the night before and each night thereafter for the duration of the study. Both had a cleansing enema an hour before and emptied the bladder just before the dose was given. Blood samples were taken 3 times the first day and twice thereafter. The entire urine output for each 12-hour period was collected for 5 days and all stools were saved *in toto* for 6 days. The method of Bratton and Marshall[5] was used for the carboxythiazole determinations. The drug content of the feces was determined as follows: the total contents of each stool were immersed in water and the mixture was made alkaline with concentrated NaOH, boiled, homogenized, made up to a given volume (usually 500 to 1500 cc) with water, strained through several layers of gauze, and an aliquot was treated like blood samples for the sulfonamide determination.†

The study was later repeated, each subject this time being given an initial dose of 4 g followed by 1 g every 4 hours until a total of 29 g were given in 100 hours. Blood samples were obtained daily for 5 days and all urine and feces were collected for 10 to 13 days. During this study, too, Subject A was given a laxative (cascara sagrada) each evening.

Four patients with bacillary dysentery and one with acute gastro-enteritis were treated with carboxythiazole. Each was given 4 g followed by 1 g every 4 hours, the former for 8 to 15 days and the latter for 3 days. Cultures of the stools were made frequently before and after the treatment was started and until the patient left the hospital.‡ Blood levels of carboxythiazole were determined daily.

Results. The results obtained following the ingestion of 5 g of carboxythiazole are shown in Table I. The amount of the drug recovered from the urine was greatest in the first 12 hours in both subjects. Only traces, however, were detectable in the blood samples obtained during this time or in the later ones. The largest amount was recovered from the first stool passed after 27 hours in Subject A who was receiving a daily laxative. In Subject B, whose bowels were sluggish, only small amounts were found in the stools during the first 3 days, and the largest amounts were recovered from the stools passed 84 and 107 hours after the dose was given. Some of the drug was still present in the urine on the fifth day and in the stool on the sixth day, the amounts being larger at these times in B than in A.

5 Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

† The chemical determinations were carried out by Ellen J. Doyle and Clare Sacco.

‡ The bacteriological studies were carried out by Miss Marion E. Lamb and her associates at the Mallory Institute of Pathology.

TABLE II.
Recovery of Carboxythiazole from Urine and Feces after Oral Administration of 4 Grams Followed by 1 Gram Every 4 Hours for a Total of 29 Grams.

Subject A						Subject B					
Urine			Feces			Urine			Feces		
Hr after initial dose	Free mg	Total mg	Hr after initial dose	Free mg	Total mg	Hr after initial dose	Free mg	Total mg	Hr after initial dose	Free mg	Total mg
0-24	239	235	0-5	224	224	0-24	153	176	0-6	100	138
24-48	262	306	5-10	3754	3816	24-48	352	404	6-26	80	128
48-72	360	401	10-33	1238	1238	48-72	410	502	26-36	228	242
72-96	322	372	33-37	1420	1420	72-96	494	548	36-53	95	95
96-120	218	218	37-77	4508	4508	96-120	257	257	53-62	280	315
120-144	162	179	77-105	6702	7579	120-144	381	461	62-98	80	88
144-168	120	149	105-171	2924	3060	144-168	281	328	98-108	231	231
168-192	118	137	171-216	39	45	168-192	178	243	108-130	3917	3917
192-216	31	64	216-241	65	68	192-216	159	213	130-150	3156	3156
216-240	5	20				216-240	73	100	150-179	2240	2378
240-264	5	12				240-264	70	96	179-194	2346	2394
264-288	0	0				264-288	58	64	194-217	722	850
						288-312	0	13	217-242	1196	1538
									242-265	560	568
									265-291	94	94
									291-315	28	28
0-288	1842	2093	0-241	20892	21958	0-312	2866	3405	0-315	15353	16160
Conjugated 12%				5%			16%			5%	

Total amt recovered = 24.051 g = 82.9%
Blood levels 4, 26, 52, 73, and 100 hrs after initial dose all <1 mg%.

19.565 g = 67.5%
Blood levels 4, 26, 52 and 73 hours after initial dose = <1 mg%; after 100 hrs = 1.4 mg% (free and total).

The total amount recovered in A (69.6% of the administered dose) was greater than in B (54.6%). Of these total recoveries 12% was obtained from the urine in A as compared with 22% in B. Only 2-3% of the amount recovered from the feces was in the conjugated form. In the urine, on the other hand, 19% of the drug recovered in A and 30% in B were in this form.

The results obtained with repeated doses are shown in Table II. In both of the subjects only traces of drug were found in the blood except in the sample taken at the time of the last dose in Subject B which showed a level of 1.4 mg per 100 cc. In both subjects, the amounts recovered from the urine were greatest during the third and fourth day of the drug administration and then decreased progressively after the last dose. With respect to the amounts of drug recovered from the feces, the 2 subjects differed as they did after the single dose. Large amounts were recovered from the feces on the first day in Subject A who was receiving daily laxatives, whereas, in B large amounts were not recovered from the feces until the sixth day. Only small amounts were recovered from urine and stools beyond the fourth day after the last dose was given in A while fairly large amounts were still present in both the urine and feces collected on the seventh day after the last dose in B.

In this instance, too, the total amount of drug recovered was greater in A (82.9% of the administered drug) than in B (67.5%). Of this amount only 9% was found in the urine in A as compared with 17% in B. Of the amount of drug recovered in the feces, 5% was determined as the "conjugated" form in both subjects while 12% of the drug recovered from the urine in A and 16% in B were found in this form.

The 5 patients treated with carboxythiazole were only moderately ill, afebrile and having 3 or 4 loose bowel movements a day when the treatment was started. Each of the 4 patients with dysentery previously had 2 to 4 positive stool cultures, strains of Sonne (Duval) being

obtained in 3 cases and of Flexner dysentery bacilli in the fourth. The fifth patient had an unidentified pathogen in the stool obtained before the treatment was begun. All stool cultures were negative for pathogens after 1 day of treatment in 2 of the cases with the Sonne strains and in the case of acute gastro-enteritis, while in the third case of Sonne infection and in the case of Flexner dysentery positive cultures were obtained during the first 2 days of treatment and all the cultures taken on the following day and thereafter were negative for pathogens.‡

The total dose of carboxythiazole was 46-81 g (average 64) in the dysentery cases and was given over an average of 10 days. The case of acute gastro-enteritis received 22 g in 3 days. Daily blood samples showed only traces of the drug in almost every instance. In one case a blood level of 2.0 mg% was obtained on the eleventh day and in another a level of 1.2 was obtained on the second and eighth days. In each of 2 other patients levels of 1.5-5.1 were obtained in the first 2 morning samples of blood after the night nurse had given 2 or 3 doses of sulfathiazole by error.

Clinically, the patients were improved and the diarrhea subsided in each case within 1 or 2 days after the treatment was started. No toxic reactions of any sort were encountered in these patients or in the experimental subjects.

Discussion. These observations in humans confirm the findings in animals that carboxythiazole is poorly absorbed after oral administration and as far as could be determined from these few trials, is non-toxic. The failure to obtain positive cultures for the pathogenic strains after its use also suggests that it may be effective and deserves a place with the other drugs used in intestinal infections. Its relative value as compared with other drugs now used for this purpose would require an extensive clinical trial under controlled conditions.

Of interest is a comparison of the findings in Subject A who received laxatives daily and in Subject B who had rather sluggish bowels. In both of them the maximum urinary excretion of the drug occurred within a few hours after it was ingested. In the former, however, the drug appeared rapidly and in large amounts in the stools, whereas large amounts did not appear in the stools of B until after the third day. An appreciably greater percentage of the administered drug was recovered from A than from B after both the single dose and the multiple doses and there was less conjugated drug in the urine of A in each instance. In addition, the amount of carboxythiazole recovered from the urine in A constituted a much smaller proportion of the total drug recovered than was the case in B. These findings are in keeping with the prolonged though slight absorption of the drug in B which permitted a greater urinary output and also a longer time for conjugation to occur. Only a small part of the drug found in the feces was determined in the conjugated form.

It seems likely that there was still an appreciable amount of drug in the bowel after the completion of the studies particularly in Subject B, and this may have accounted for the smaller percentage of the administered drug which was recovered in that case. The possibility that part of the drug is destroyed in the body cannot be excluded.

Conclusions. "Carboxythiazole" (2-sulfanilamido-5-carboxythiazole) is poorly absorbed after oral administration to man and produced no toxic symptoms in 5 patients receiving 4 g initially and 1 g every 4 hours for 3-15 days. The drug may be useful in the treatment of enteric infections and in bowel surgery.

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Distribution of Proteoses and Peptones After Intravenous Injection.

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This investigation was concerned with the distribution of proteoses and peptones in the mouse after intravenous injection. The metabolism and distribution of amino acids labeled with deuterium and N^{15} after oral and parenteral administration have been studied by Schoenheimer, Rittenberg, *et al.*[1] Freund and coworkers[2] determined the changes in the composition of non-protein nitrogen after intravenous injection of peptones and proteoses. They concluded that the peptones were absorbed into the intestine. Fine *et al.*[3] injected proteins containing radioactive elements (Br or S) into animals in shock. The rate of disappearance of radioactivity varied with the protein depending on the method by which the radioactive element was introduced.

In this work a pepsin digest of yeast containing N^{15}, uniformly distributed, was used. The distribution of the injected material was determined from the nitrogen and N^{15} analyses.

Materials. Yeast (*Saccharomyces cerevisiæ*) was grown in a synthetic medium (Frey* *et al.*[4]) in which the only source of nitrogen was ammonium chloride or sulphate containing 10 or 30% N^{15}. After 24 hours at 30°C with slow, continuous, and mild shaking, the yeast was centrifuged, washed repeatedly with water, acetone, and ether and finally dried in vacuum over sulphuric acid. Meisenheimer[5] has shown that 7.5 to 9.6% of yeast nitrogen is purine nitrogen and identified 15 amino acids which accounted for another 74% of the nitrogen. The presence of additional amino acids in yeast protein has been demonstrated more recently.[6]

The dried yeast was suspended in water and digested at pH 2 and at 40°C with a concentrated pepsin solution for two hours. The digest was neutralized and centrifuged. The supernatant was used for the injections. The insoluble residue contained carbohydrates and 16 to 18% of the original nitrogen. Peptones and proteoses were determined in the supernatant liquid as follows: 10 ml of the material, containing 0.2% nitrogen and 10% sodium sulfate, was acidified with acetic acid to pH 5 and one ml of a freshly prepared 10% tannic acid solution added. The solution was mixed thoroughly, kept for 24 hours at 0°C, and centrifuged at 0°C. The nitrogen content of the supernatant solution was determined. On the average 62% of the total nitrogen was precipitated by tannic acid, which compares favorably with the results of Wasteneys and Borsook[7] on pure peptone solutions. It may be assumed that at least 62% of the nitrogen in the supernatant consisted of peptones and proteoses.

Experimental. The solution was injected into the tail veins of fasting male albino mice weighing from 20 to 25 g. The peptone used in mice No. 1 and No. 3 of Table I contained 9.03% N^{15}, in all others 27.0% N^{15}.

The mice were killed by decapitation 10, 30,

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[2] Freund, E., *Z. exp. Path. Ther.*, 1907, **4**, 1; Freund, E., and Popper, H., *Biochem. Z.*, 1909, **15**, 272.

[3] Fine, J., and Seligman, A. S., *J. Clin. Invest.*, 1943, **22**, 265 and 285.

* We are indebted to Dr. Frey for the yeast strain and the personal communication of the details of the growing technic.

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[5] Meisenheimer, J., *Z. physiol. Chem.*, 1910, **104**, 229; 1921, **114**, 205.

[6] Block, R. J., and Bolling, D., *Arch. Biochem.*, 1943, **3**, 217; Carter, H. E., and Phillips, G. E., *Fed. Proc.*, 1944, **3**, 123.

[7] Wasteneys, H., and Borsook, H., *J. Biol. Chem.*, 1924-5, **62**, 1.

TABLE I.
Distribution of N^{15} After Peptone Injection.

Mouse: / Killed after: / Injected	No. 1, 22 g 10 min 1.0 ml		No. 8, 21 g 10 min .15 ml		No. 20, 23 g 10 min .25 ml		No. 3, 20 g 30 min .8 ml		No. 10, 24 g 60 min .22 ml		No. 21, 24 g 60 min .25 ml		No. 11. 23 g 180 min .25 ml	
Organs					A. % Excess N^{15}.									
Liver	.014		.018		.038		.025		.027		.057		.021	
Gastro-Intestinal Tract	.019		.031		.021		.035		.033		.109		.034	
Blood, Spleen and Heart	.009		.022		.014		.018		.018		.034		.018	
Lung	.025		.017		.016		.031		.017		.034		.080	
Kidney	.028		.086		.030		.055		.018		.062		.113	
Uro-genital Tract	.191		.056		.067		.121		.019		.101		.689	
Skin	.053		.012		.071		.090		.026		.047		.029	
Carcass	.024		.011		.057		.032		.012		.036		.018	
Tail	.013		.063		.068		.016		.086		.045		.062	
Excreta	*		*		*		*		.598		.527†		.016	
Total excess N^{15} found in atoms $\times 10^{-6}$	12.2		7.2		26.0		11.7		12.3		24.4		14.3	
	B. Distribution of the N and N^{15} in % of the Total N and N^{15} of the Animal.													
	N	N^{15}	N	N^{15}	N	N^{15}	N	N^{15}	N	N^{15}	N	N^{15}	N	N^{15}
Liver	6.0	3.1	5.1	5.5	5.2	3.8	8.3	6.0	6.2	6.0	4.4	4.8	5.0	3.4
Gastro-Intestinal Tract	9.1	6.0	10.0	17.2	8.6	3.5	9.1	9.4	8.8	11.5	8.2	17.6	8.3	8.7
Blood, Spleen and Heart	4.6	1.4	3.5	4.2	6.0	1.7	5.0	2.6	4.6	3.0	3.9	2.7	4.4	2.7
Lung	0.9	0.6	0.7	0.8	0.7	0.2	0.8	0.6	0.7	0.5	0.6	0.5	0.7	1.7
Kidney	1.3	1.2	1.4	0.7	1.6	0.9	1.6	2.5	1.5	6.0	1.4	1.7	1.5	5.8
Uro-genital Tract	1.0	6.6	1.1	3.8	2.8	3.6	1.5	9.2	2.6	1.8	0.9	1.2	2.6	1.4
Skin	22.0	38.3	21.0	16.3	17.0	23.2	19.3	22.2	23.0	21.8	25.2	23.2	27.8	20.3
Carcass	50.2	41.8	53.0	37.3	54.4	58.2	50.7	45.8	48.3	27.0	51.3	35.2	51.0	29.3
Tail	4.9	2.2	4.2	14.0	3.8	4.9	3.7	1.7	4.3	13.7	4.2	3.9	3.7	7.7
Excreta	—	—	—	—	—	—	—	—	—	8.7	-	9.2	—	18.9

* No excreta.
† Urine only

60, and 180 minutes after injection. The bodies were immediately divided into the following fractions: 1, liver; 2, gastro-intestinal tract; 3, blood, spleen, and heart; 4, lungs; 5, kidneys; 6, urogenital tract (without kidney); 7, excreta; 8, skin; 9, carcass; 10, tail. Each fraction was digested with sulphuric acid and the total nitrogen determined. The N^{15} of each fraction was determined according to Rittenberg, Schoenheimer, *et al.*[8] The experimental error of the method is $\pm 0.003\%$ N^{15}. In these experiments all values up to 0.008% excess N^{15} were considered as not significant and values 0.09% to 0.10% as traces. The total N^{15} recovered was within 10% of the amount injected.

Table I-A gives the values for N^{15} concentration of each fraction at 10, 30, 60, and 180 minutes after the injection. Table I-B presents the distribution of nitrogen and N^{15} for each fraction in per cent of total recovered nitrogen and N^{15}.

Within 10 minutes after injection, N^{15} is rapidly removed from the blood stream and deposited primarily in skin and muscles (54-81%). Blood, spleen, and heart accounted for 2.6-3.0% of the injected N^{15}. The highest concentration of N^{15} was present in the urogenital fraction, although the total amount of N^{15} was low.

During the course of the experiments, the concentration and amount of N^{15} decreased in the carcass and gradually increased in kidneys, urogenital tract, and excreta, 8.7%-9.2% of N^{15} being excreted after one hour and 18.9% after 3 hours. The amount of N^{15} in blood, spleen, and heart remained low (2.6-3.9%), throughout the experiment. The gastrointestinal tract showed an increase of N^{15} after 30 minutes and one hour, and a decrease after 3 hours.

To establish the form in which N^{15} was excreted in the urine (see Table I, mouse No. 21), 2 groups of 3 fasting male mice (weight 20-25 g) received intravenously .1 ml of a 2% yeast peptone solution. Two hours after the injection, the urine of each group together with the washings was collected and brought to 1 ml. To remove the coloring matter 0.1 ml of a 10% lead acetate solution was added, the mixture heated 5 minutes at 100° and centrifuged.[9] Excess lead was removed from the supernatant fluid by careful treatment with aqueous sodium hydroxide. The solution gave a negative biuret reaction and no precipitate with tannic acid at pH 5 and 0°. Normal human urine containing 0.50% to .025% added peptone treated in the same way gave a positive biuret reaction.

Discussion. The relatively high concentration of N^{15} in skin and carcass as well as the presence of between 50 and 80% of the total N^{15} in these organs seems to indicate a depot function for these organs in nitrogen metabolism. This is indicated at least for proteoses and peptones, since no similar distribution for amino acids was found by Schoenheimer *et al.*[1] The short period (10 minutes) of the first experiment does not seem to be sufficient time to allow for much extensive alteration of the injected materials and it may be assumed that they were absorbed or deposited as such. Similar high values at after one and 3 hours for skin and carcass would corroborate this conclusion. Nevertheless transamination or breakdown of the injected material and resynthesis of the amino acids into body protein may have occurred. It is interesting to note that the N^{15} of the carcass decreases and remains fairly constant for the skin. This is more notable if the distribution of the total nitrogen and N^{15} are compared for each fraction. The N^{15} concentration in the skin is always higher than in the carcass. This would be consistent with the picture of the carcass as an active storage organ and the skin with a less active function.

There is also evidence for absorption of injected N^{15} into the gastrointestinal tract throughout the experiment. Comparison of the average amount of total nitrogen and N^{15} for each fraction after different time periods, indicates an increase in the N^{15} concentration in the liver and especially in the gastrointestinal tract during the first 60 minutes. After 3 hours both fractions especially the liver show a decrease in the concentration.

Summary. 1. Yeast peptone containing

[8] Rittenberg, D., Keston, A. S., Rosebury, F., and Schoenheimer, R., *J. Biol. Chem.*, 1939, **127**, 291.

[9] Freund, O., *Centr. inn. Med.*, 1901, **22**, 27.

excess N^{15} was injected intravenously into mice. 2. The N^{15} was rapidly removed from the blood stream and after 10 minutes was found principally in the skin and carcass. The remainder of the N^{15} was accounted for in the gastrointestinal tract, liver, and urogenital tract. 3. Thirty to 180 minutes after injection the predominant portion of the N^{15} still appeared in the skin, carcass, gastrointestinal tract and with progressing time in the excreta. 4. On the average the N^{15} in the carcass showed a decrease after one hour, while the gastrointestinal tract showed an increase during the first hour and a decrease after 3 hours.

The interest and helpful criticism of Dr. Rittenberg are gratefully acknowledged.

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Rate of Flow and Cell Count of Rat Thoracic Duct Lymph.*

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In the course of studies on the action of adrenocorticotrophic hormone on the leucocyte count in the rat,[1] it was thought advisable to study directly the effect of hormone administration on the lymphocyte cell content of thoracic duct lymph in this animal form. Review of the literature did not, however, reveal any description of the technic of cannulation and collection of lymph in the rat. The present report describes a method of cannulation of the thoracic duct in the rat, and observations on the white cell count, and rate of flow of lymph in the normal, adult female rat.

Technic. Rats of the Long-Evans strain, of weight 200-250 g, unfasted, were employed. Preliminary studies indicated the difficulty of accurate identification of the lymphatic trunks in the neck so that it was necessary to identify the vessels by intraperitoneal injection of ½ cc of 1% trypan blue, half an hour before cannulation was attempted. This amount of dye was sufficient to stain all the main lymphatic trunks and to demonstrate the site of entrance of the lymph stream into the junction of internal jugular and subclavian veins with the left superior vena cava (in the rat). When familiarity with the lymphatic vessel arrangement was attained, further use of the dye was obviated.

The animals were anesthetized by intraperitoneal injection of 50-60 mg/kg B.W. of sodium pentobarbital in 1% solution. Further amounts were injected as necessary to maintain the anesthetic state over long periods of time.

Two types of cannulæ were employed. Capillary tubes were used for drop-wise collection of the lymph. In other cases, 3 to 5 mm glass tubing was drawn out into a capillary tube at one end for the collection of larger samples of lymph. The capillary tubings were drawn to the size, externally, of No. 25 G-No. 27 G hypodermic needles, which were employed to make openings in the lymph vessels previous to insertion of the cannula. All manipulations and cannulation were carried out under the binocular dissecting microscope. Cannulæ were heparinized to prevent clotting. A midline incision was carried through skin and subcutaneous tissue of the neck, through the deep fascia, separating the submandibular salivary glands, dissecting to the ventral aspect of the sternohyoid muscles. Using a small pair of bone forceps, the manubrium sterni was split in the midline as far as the sternal angle. The two halves of the manubrium were retracted laterally by means of clamps and held apart exposing the length of

* Aided by grants from the Research Board of the University of California and the Rockefeller Foundation, New York City.

1 Reinhardt, W. O., Aron, H., and Li, C. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 19.

the sternal attachment of the sternohyoid muscles. The left sternohyoid muscle was bluntly dissected away from its attachments to the manubrium, exposing the site of juncture of the left superior vena cava and the internal jugular vein. Lying in close dorsal approximation to the internal jugular vein is the small lymph sac formed by the thoracic duct, its jugulo-subclavian tributaries, and the site of union with the venous system. The main cervical lymph trunk was identified and followed down to the point at which the cervical trunk joined the thoracic duct. A No. 27 G hypodermic needle was thrust through the wall of the cervical lymph vessel, and the cannula was inserted into the vessel and threaded down the thoracic duct. Lymph rushed into the cannula immediately and could be collected by any desired method, either drop-wise for lymph cell counts or in larger quantities for chemical analysis.

In some experiments, cannulæ were inserted directly into the cervical lymph trunk and threaded into the thoracic duct. It is undoubtedly possible to cannulate and collect lymph from any of the tributaries of the thoracic duct or lymph sac. It may be noted that the living lymph vessels are very thin-walled and practically transparent, but are distensible and much larger in size than would be indicated by embalmed animal dissections.

Results. Under the binocular microscope, clear lymph of the cervical and subclavian lymph trunks may be seen mixing with the usually milky and opaque thoracic duct lymph prior to entrance into the venous system. The rate of flow of thoracic duct lymph varied from 0.13-0.7 cc per hour, averaging 0.45 cc in 10 animals. In one animal, 0.7 cc were collected in an hour; in another, 2.0 cc in 6 hours.

On the basis of a thoracic duct lymph flow of 0.5 cc/hr, it can be calculated that a volume of at least 12.0 cc of thoracic duct lymph passes into the venous blood stream/24 hours. This amounts to an estimated 75% of the total blood volume (6.5% of total B.W.) in rats of the size employed.

White cell counts were carried out on the thoracic duct lymph of 17 rats. The total white cell count averaged 19,050, and varied from 6,650-46,250 cells per cu mm. Supravital studies and Wright-stained differential cell counts disclosed that the white cells were almost uniformly small and medium sized lymphocytes.

The above values correspond closely to those for other animal forms studied.[2]

It should be remarked that the amount of lymph available on cannulation of the rat thoracic duct is more than sufficient to carry out most morphological or chemical studies.

Summary. 1. A technic is described for obtaining lymph from the thoracic duct of the rat. 2. The average rate of flow of thoracic duct lymph in the adult, female, unfasted rat, anesthetized with sodium pentobarbital was approximately 0.45 cc/hour (range: 0.13-0.7 in 10 rats). 3. The white cell count of the thoracic duct lymph in 17 rats varied from 6,650-46,250 cells/cu mm and averaged 19,050 cells/cu mm. 4. The rat is a useful and convenient animal to employ in studies on physiology of the lymph.

[2] Drinker, C. K., and Yoffey, J. M., *Lymphatics, Lymph and Lymphoid Tissue*, Harvard University Press, Cambridge Mass., 1941.

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Malarial Infections in the Duck Embryo.

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Infection of duck embryos with *Plasmodium cathemerium, P. lophuræ,* and *P. elongatum* has been reported.[1] In the present work only the first two species were used in attempts to produce standardized, highly-reproducible infections. After intraembryonic inoculation of infected duck blood the 11- to 16-day duck embryos were incubated at 37°C or 40°C. Inoculation doses ranged from about 12 to 50 million parasitized cells per embryo. (Intravenous injection of such numbers of either species of *Plasmodium* into ducklings produces infections with no prepatent period. A high immediate mortality (50%) within 2-3 days follows intraembryonic injection of as little as 0.05 ml of any material.) A total of 72 embryos was inoculated after various preliminary experiments.

A prepatent period of variable length occurred in the embryo. This depends probably on dosage, site of inoculation, temperature of incubation, and especially the species of parasite. It was consistently longer with *P. lophuræ.* This difference in rate of reproduction of the two species is true for ducklings as well as for the duck embryo. If permitted to run its course, *P. cathemerium* regularly killed the embryo in from 6-11 days with evidence that a high degree of parasitemia occurred (up to 50% of the erythrocytes). With inocula of 20 to 25 million parasitized cells per embryo and incubation at 37°C death occurred regularly on the 10th or 11th days. Both species can develop in the embryo at either 37°C or 40°C. The earliest embryonic deaths with *P. cathemerium* occurred at the higher temperature. The *P. lophuræ* infection developed more slowly and even 14 days after inoculation did not show the intense parasitization of the embryo regularly seen in the duckling. For the purposes of this work *P. lophuræ* therefore was not a satisfactory parasite.

An embryo of 19 days incubation still shows an occasional primitive erythrocyte of yolk sac origin. Two embryos of this age exposed to similar temperatures of incubation, size of inoculum and level of parasitization (18 and 27% respectively) were examined in detail. The one, infected with *P. cathemerium,* revealed only an 11% infection of the primitive erythrocytes while in the other, infected with *P. lophuræ,* 76% of the rare primitive red cells were parasitized. This differential rate of infection of erythrocytes probably is confirmatory evidence of the influence of the age of the erythrocyte on its susceptibility to penetration by plasmodia.[2] In one embryo elongated gametocytes of *P. cathemerium* were seen, some with jagged edges, such as those described as occurring in ducks infected with *P. relictum.*[3]

As in the duckling, infection of the embryo with either species led to splenomegaly and malarial pigmentation. Separating the spleens of those embryos uninfected, dying or killed early in the infection from those dead of malaria or killed late in the patent period (only *P. cathemerium* data were sufficient) we found that it is unlikely that a spleen of gross size more than 19 mg is from an uninfected embryo even when the embryo is 18 or more days old. Spleen weight-body weight ratios ranged from 1.2 to 2.4 mg per g in the heavily infected embryos (the mean of 9 determinations was 1.8 mg/g). In the other group the mean ratio of 13 specimens, some of which had been incubated more than 24 days, was only 0.7 mg/g with only one specimen as large

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1 Wolfson, F., *Am. J. Hyg.,* 1940, **31**, Sec. C, 26.

2 Hewitt, R., *Bird Malaria,* Baltimore, 1940.

3 Wolfson, F., *Am. J. Hyg.,* 1939, **30**, Sec. C, 123.

as 1.2 mg/g. Fatty changes, deposits of bile pigment and erythropoiesis in the liver were additional features of the pathological picture encountered only in infected embryos.

Having established that reproducible malarial infections of the duck embryo could be obtained with inoculations of erythrocytic stages of *P. cathemerium*, we undertook a preliminary study of the possibility of producing infections using the ground organs of infected mosquitoes (*Culex pipiens*).‡ Though no infections have been produced as yet some embryos can survive at least 14 days after such inoculation when incubated at 40°C. No attempt was made to produce sterile inocula beyond the use of completely sterile equipment.

‡ The strain of *Culex pipiens* was kindly supplied by Dr. F. Wolfson.

Summary. Intraembryonic inoculation of duck embryos with the 3T strain of *P. cathemerium* causes reproducible fatal infections with a high level of parasitemia 6-11 days after inoculation. Splenomegaly occurs in these infected embryos. Intraembryonic inoculation of *P. lophuræ* produces much more variable results. Infection requires a considerably longer period to develop. This may depend upon the maturity of the erythrocyte in the embryo. Both species of plasmodia can develop in the embryo at either 37°C or 40°C. Duck embryos can survive the intraembryonic inoculation of ground organs of malaria-infected mosquitoes if they are incubated at 40°C.

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Relative Susceptibility of Staphylococci to the Bacteriostatic Action of Antibiotics.

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During the last few years many antibiotics have been studied experimentally and several, notably penicillin, tyrothricin, streptothricin, and streptomycin, have been employed successfully in the treatment of human infections or are being investigated at the present time regarding their clinical usefulness. It is well known that the spectra of activity vary from compound to compound. Certain antibiotics, however, are more effective than others against some species of microörganisms. Herrell and Heilman[1] found penicillin superior to gramicidin in bactericidal activity toward staphylococcus, pneumococcus, and hemolytic streptococcus. The question presents itself as to whether or not various strains belonging to one and the same species differ in their susceptibility to the action of antibiotics. Crowe and Ward[2] state that certain strains of staphylococci are insensitive to tyrothricin or crude penicillin. On the other hand, Rammelkamp and Maxon[3] reported that 29 different strains of staphylococci varied only slightly in their resistance to penicillin. The results of an investigation into the relative activity of penicillin, tyrothricin, and streptothricin on various strains of staphylococci are embodied in the ensuing report.

Material and Methods. Strains of *Staphylococcus aureus*, isolated from patients at this hospital, were used in this investigation. In addition, several strains (Nos. 3, 20, 209) were obtained through the kindness of Dr. Jean V. Cooke, Washington University, St.

1 Herrell, W. E., and Heilman, D., *Am. J. Med. Sci.*, 1943, **206**, 221.

2 Crowe, S. J., and Ward, A. T., *N. Y. State J. Med.*, 1945, **45**, 61.

3 Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

Louis. The strains were kept on brain veal agar. For the experiments, they were grown in brain heart infusion for 18 hours at 37°C. Suspensions, containing approximately 10,000,000 organisms per ml, were prepared for seeding purposes.

All antibiotics were dissolved in brain heart infusion. Sodium penicillin-broth was freshly prepared for each experiment from a stock solution containing 100,000 U per 10 ml. This stock solution was kept in the refrigerator for 3-4 days. Streptothricin was supplied through the courtesy of Merck & Co. A stock solution containing 100 mg% of this compound was prepared and sterilized by filtration; it was kept in the refrigerator and dilutions thereof were prepared freshly for each experiment. Brain heart infusion containing various concentrations of tyrothricin was made from a stock solution containing 25 mg per ml of this compound.

The experiments were set up as follows: To 2 ml of broth containing various amounts of antibiotics, was added 0.2 ml of the staphylococcal suspension. Broth, used as a control, was seeded likewise. The broths were then incubated at 37°C. The resulting growth was noted grossly at various intervals.

Results. Experiments with various strains of coagulase-positive and coagulase-negative staphylococci, revealed that the relative susceptibility to the bacteriostatic action of penicillin, streptothricin, and tyrothricin varied from strain to strain. The results of the illustrative experiment carried out with four selected coagulase-positive strains are presented in Table I.

It may be noted that strain No. 1725 was less susceptible to penicillin and more susceptible to tyrothricin than strain CH. The former strain was more resistant to penicillin than all others studied. Strain No. 3 was less susceptible to streptothricin and more susceptible to penicillin than strain No. 1725. Strain No. 2019 was inhibited in its growth by all three antibiotics in the concentrations employed and thus differed from the other 3 strains. These findings were corroborated in repeated experiments. It is evident, therefore, that the 4 strains of *Staphylococcus aureus* differ in their relative susceptibility to

TABLE I.
Bacteriostatic Action of Various Antibiotics on Staphylococci.

Antibiotic substance		Strains of Staphylococci							
		No. 1725		No. CH		No. 2019		No. 3	
		a	b	a	b	a	b	a	b
Penicillin	1 U/ml	—	++++	—	—	—	—	—	—
	0.1 ,, ,,	++++	++++	—	—	—	—	—	—
Streptothricin	20 mg%	—	—	—	++	—	—	—	++++
	10 ,,	—	++++	++++	++++	—	—	++++	++++
Tyrothricin	2.5 ,,	—	—	—	—	—	—	—	—
	0.8 ,,	—	—	++++	++++	—	—	—	—
Control		++++	++++	++++	++++	++++	++++	++++	++++

a = 24 hr at 37°C. b = 96 hr at 37°C.
— = No visible growth. + to ++++ = Various degrees of visible growth.

these antibiotics.

Similar results were obtained with other strains of staphylococci. For instance, strain No. C proved to be less susceptible to tyrothricin and markedly more susceptible to both penicillin and streptothricin than strain No. 1725. The growth of strain No. 1725 was not inhibited in the presence of 1 U of penicillin, whereas that of strain No. 2535 was prevented by 0.1 U of this antibiotic. In contrast, tyrothricin in a concentration of 0.25 mg% prevented the growth of strain No. 1725 for 24 hours and failed to do so with strain No. 2535 in a concentration of 0.6 mg%. Of 3 selected coagulase-negative strains No. 2320 was more susceptible to the action of penicillin than Nos. 2203 and 2204.

Quantitatively, a certain strain of staphylococcus may grow in the presence of an antibiotic in concentrations from 2 to 10 times greater than that required to prevent visible growth of another strain. It is evident that according to the results of these experiments, the relative susceptibility or insusceptibility of a certain antibiotic does not necessarily parallel a similar susceptibility or insusceptibility to another antibiotic.

Discussion. The observations reported here seem to indicate that the mode of action of the antibiotics tested is not alike. As yet it is not possible to state which properties of different strains of staphylococci determine the relative susceptibility or insusceptibility to the action of these drugs. Furthermore, it is conceivable that the findings may have clinical implications, provided that the difference in susceptibility of various strains observed *in vitro* manifest themselves also *in vivo*. If this be the case, it may be advisable under certain circumstances to determine *in vitro* the relative susceptibility of microörganisms to various antibiotics as the basis for the selection of the most suitable chemotherapeutic agent.

Summary. Various strains of staphylococci differ in their relative susceptibility to the bacteriostatic action of penicillin, tyrothricin, and streptothricin *in vitro*. The relative susceptibility to one antibiotic is not necessarily paralleled by a similar susceptibility to another antibiotic. The implications of these observations are discussed.

Sincere thanks for technical assistance are expressed to Miss Elizabeth C. Deuchler.

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2(Naphthyl-(1')-Methyl)-Imidazoline Hydrochloride (Privine).* I. Potency in Modifying Cocaine Hydrochloride-Induced Convulsions.

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The early work of Braun,[1] confirmed by Maurel,[2] Custer,[3] and others, seemed to establish that epinephrine simultaneously injected subcutaneously with cocaine exerted its protective action against cocaine-induced convulsions in a mechanical fashion by constricting the vicinal vessels and so delaying the absorption of the cocaine that a convulsive level was never attained in the blood stream. It occurred to us that advantage might be taken of this phenomenon to compare the effectiveness of different vasoconstrictors. As the first phase of an extended study of the vasoconstrictor properties of Privine we employed this method of comparing its effectiveness with that of epinephrine.

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† Now with the Department of Biochemistry, the University of Rochester School of Medicine and Dentistry, Rochester, N.Y.

1 Braun, H., *Arch. f. klin. Chir.*, 1903, **69**, 541.

2 Maurel, E., *Bul. gen. de Therap. Paris*, 1892, **122**, 201.

3 Custer, *Kokain and Infiltrations Anästhesie*, Basel, 1898, p. 34.

TABLE I.

Cocaine Convulsions in Guinea Pigs as Modified by Epinephrine or Privine.

Each animal received 1.0 cc/kg of 4% cocaine subcutaneously. Incorporated with the cocaine was the indicated concentration of vasoconstrictor. Time is expressed in min.

Vasoconstrictor	No. of animals	Avg time before first convulsion	Avg time before death	% convulsed	% dead*	% protected from convulsions
			Cocaine Control			
0	10	8 (7-11)†	16 (12-19)	100	50	0
			Epinephrine			
1:200,000	7	10 (5-20)	23 (16-37)	100	57	0
1:100,000	10	10 (3-17)	32 (1)	20	10	80
1: 50,000	10	6 (4- 8)	11 (1)	60	10	40
1: 25,000	26	13 (6-25)	15 (11-17)	54	15	46
1: 10,000	10	7 (4-12)	14 (11-19)	50	30	50
1: 5,000	5	4 (2- 5)	9 (6-15)	100	100	0
			Privine			
1:200,000	7	7 (3-10)	12 (7-18)	100	57	0
1:100,000	10	6 (3- 9)	14 (11-20)	90	60	10
1: 50,000	10	9 (6-13)	14 (11-17)	100	50	0
1: 25,000	10	7 (6-12)	17 (11-29)	80	50	20
1: 10,000	16	6 (2-14)	11 (5-17)	100	88	0
1: 1,000	10	5 (2- 9)	8 (4-16)	100	90	0
			Privine Control (without cocaine)			
1: 1,000	10				0	
1: 250	10				0	
1: 100	10		95 (59-148)		100	

* This includes animals dead within an hour. An occasional animal dying overnight has been omitted.

† The figures in parentheses represent the extreme values.

Methods. Guinea pigs in the weight range 275-325 g were employed for this study in order to minimize the effect of weight as a variable because we early established that the toxicity of cocaine was relatively greater for lighter animals. Guinea pigs in this weight range all convulsed after the subcutaneous injection of 1 cc of 4% cocaine per kg. Various concentrations of each vasoconstrictor were prepared and sufficient cocaine was added to each to make the solution 4% in respect to cocaine. The effectiveness of each concentration of the vasoconstrictor was then judged by its ability, when injected with the cocaine (1 cc of 4%), to prevent convulsions or to delay their onset. The occurrence or absence of convulsions in guinea pigs injected with 1 cc of 4% cocaine per kg was more desirable as an end-point than death for two reasons: (1) it was more economical and (2) the animals either developed convulsions within the first hour or never did. Some animals, on the other hand, died many hours after the injection, but these animals had always convulsed during the first hour. The results are summarized in Table I.

Discussion. This method gave a rough comparison of the relative effectiveness of the two drugs in causing subcutaneous capillary constriction but the limited number of animals did not seem to warrant a statistical analysis.

The most effective concentration of epinephrine, not only for diminishing the incidence of convulsions but for saving the lives of the animals, lay between 1:100,000 and 1:25,000. This finding may have some clinical significance. A 1:5,000 concentration of epinephrine definitely augmented the toxicity of the 4% cocaine. One-half of the animals injected with 4% cocaine alone had died, whereas all died, and after shorter intervals, that had received it with 1:5,000 epinephrine incorporated therein. From these results one could not, therefore, recommend a greater concentration of epinephrine than 1:25,000 for delaying the absorption of cocaine locally injected. Higher concentrations caused the synergistic action of the drugs systematically to predominate, a conclusion reached by Ross[4] and Gold.[5] They both gave intravenous injections of cocaine to cats.

[4] Ross, E., *J. Lab. and Clin. Med.*, 1923, **8**, 657.

[5] Mayer, Emil, *J. A. M. A.*, 1924, **82**, 876; Personal communication to committee.

Privine, on the basis of this method of comparing the effectiveness of vasoconstrictors, is not comparable in potency to epinephrine. Dilutions of Privine greater than 1:10,000 killed almost the same percentage of animals as did cocaine alone. Concentrations of 1:10,000 and 1:1000 killed respectively 38% and 40% more than did the cocaine alone. The assumption of a synergism between cocaine and Privine would explain this greater mortality with the higher concentrations of Privine, although it might also be explained by the assumption that a 1:1,000 concentration of Privine caused local vascular damage or depression with a resultant dilation so that the absorption of the cocaine was actually favored. Privine in the first 4 concentrations employed (see Table I) protected a total of 3 animals from convulsions, a result of debatable significance in view of the unchanged mortality rates in those 4 groups and the insignificantly changed average time before the onset of convulsions.

The injection of 1 cc of 1:250 (4 mg) Privine per kg killed none of 10 animals; 1 cc of 1:100 (10 mg) Privine per kg killed all of 10 animals. The LD_{50} for Privine, which we did not determine, would be intermediate between those 2 doses. Baylac[6] reported the same limits for epinephrine. Animals killed by Privine exhibited that acute pulmonary edema usually seen in animals killed by epinephrine.

Summary. 1. Privine, on the basis of the method employed for comparison, is a less effective vasoconstrictor than epinephrine. 2. The LD_{50} of Privine for guinea pigs is intermediate between 1 cc of 1:250 (4 mg) and 1 cc of 1:100 (10 mg) per kilogram. 3. Actively growing guinea pigs of less than 300 g are more sensitive to a given toxic dose of cocaine in g per kg than are mature or less actively growing guinea pigs of more than 300 g. 4. The toxicity, after subcutaneous injection, of a dose of cocaine in g per kg may be decreased by decreasing the concentration of the solution used for injection; this has been previously reported by other investigators.

[6] Baylac, J., *Arch. med. de Toulouse,* 1905, **11**, 245.

14870

Method for Testing *In Vitro* Resistance of Group A Hemolytic Streptococci to Sulfonamides.*

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A suitable medium for testing sulfonamide susceptibility of Group A streptococci should meet certain requirements: it should promote good growth of all freshly isolated strains; it should promote growth from a uniform and measurable inoculum; and it should be practically free of substances antagonistic to sulfonamide action. Most media lack the last mentioned qualities. Furthermore, the relative capacity of different strains to multiply *in vitro* in the presence of sulfonamides is determined not only by inherent differences in their drug susceptibility but also by the size of the inoculum as well as by the character of the medium.

This paper describes a test in which the above mentioned criteria are fulfilled. It demonstrates the inhibition of streptococcal growth by various concentrations of sodium sulfadiazine in a semi-synthetic, semi-solid medium reinforced with serum.

The semi-synthetic medium was developed

* The opinions expressed in this paper are those of the author and do not necessarily represent those of the Navy Department.

TABLE I.
Dilution Schedule for 24 Tests.

	Control	1 mg	5 mg	25 mg	125 mg
Solution I (basal medium)	33.0	33.0	33.0	33.0	33.0
Solution V (growth factors, vitamins, and minerals)	2.5	2.5	2.5	2.5	2.5
Solution VI R or VI H	2.5	2.5	2.5	2.5	2.5
Serum	5.0	5.0	5.0	5.0	5.0
Saline (0.85% NaCl)	2.0	1.5	1.75	0.75	0.75
SSD* 0.1%	—	0.5	—	—	—
1.0%	—	—	0.25	1.25	—
5.0%	—	—	—	—	1.25
Solution VII	5.0	5.0	5.0	5.0	5.0

* SSD—Sodium sulfadiazine solutions.
All measurements are in cc.

by Roe and Adams[1] for the growth of pneumococci. It supports adequate growth of streptococci when supplemented with normal rabbit serum or with normal horse serum and xanthine. The inoculation technic was adopted from that of Ward and Rudd[2] who employed it to differentiate growth characteristics of streptococcal colonies in a semi-solid medium.

Preparation of Stock Solutions. Solution I: (Basal Medium) Caseine hydrolysate (10% solution, SMACO "vitamine free"), 200 cc; Cystine, 150 mg (dissolved in 10 cc N HCl); Tryptophane, 20 mg; KCl, 3.0 g; Na_2HPO_4 (anhydrous), 3.0 g; and $MgSO_4 \cdot 7H_2O$, 0.5 g. The ingredients are mixed in about 800 cc of distilled water; the pH is adjusted to 7.5; and the volume is made up to 900 cc. The mixture is autoclaved at 10 lbs for 10 minutes, filtered through paper, and reautoclaved.

Solution II: (Vitamins) Biotin, 0.015 mg; nicotinic acid, 15.0 mg; pyridoxin, 15.0 mg; calcium pantothenate, 60.0 mg; thiamine chloride, 15.0 mg; riboflavine, 7.0 mg; adenine sulfate, 150.0 mg; and uracil, 150.0 mg. These are dissolved in about 75 cc of distilled water; the pH adjusted to 7.2; the volume made up to 100 cc with distilled water; and the final solution sterilized by filtration.

Solution III: (Minerals) $CuSO_4 \cdot 5H_2O$, 50 mg; $ZnSO_4 \cdot 7H_2O$, 50 mg; $FeSO_4 \cdot 7H_2O$, 50 mg; $MnCl_2 \cdot 4\ H_2O$, 20 mg; and concentrated HCl, 1.0 cc; distilled water to 100 cc. This solution does not require sterilization.

Solution IV: (Growth factors) Glutamine,† 200 mg; dextrose, 2 g; Solution III, 2 cc; $CaCl_2 \cdot 2H_2O$, 10 mg; asparagin, 100 mg; choline chloride, 10 mg; and distilled water, 40 cc. Sterilized by filtration.

Solution V: (Vitamins, minerals and growth factors) Solution II, 8 cc, and Solution IV, 42 cc.

Solution VI R: $NaHCO_3$ (sterilized by autoclaving in dry test tubes), 0.2 g; sterile distilled water, 10 cc; and thioglycollic acid (10% solution, sterilized by boiling), 0.2 cc. This solution is used when the medium is to be made with rabbit serum.

Solution VI H: $NaHCO_3$ (prepared as in Solution VI R), 0.2 g; sterile distilled water, 8 cc; sterile 10% thioglycollic acid, 0.2 cc; and xanthine solution (100 mg xanthine per 100 cc N/20 NaOH, sterilized by filtration), 2 cc. This solution is used when the medium is to be made with horse serum.

Solution VII: (Agar base) Bacto agar 1.5 g is added to 100 cc of Solution I which is then heated until the agar is dissolved. After the reaction is adjusted to pH 7.5, the solution is heated until a granular precipitate forms. It is then cleared by filtering through absorbent cotton, distributed in convenient amounts and autoclaved.

Stock sodium sulfadiazine solutions containing respectively 0.1 g, 1.0 g, and 5.0 g of drug per 100 cc in distilled water are sterilized by immersion in boiling water for 10

1 Roe, A. S., and Adams, M. H., abstracted in *J. Bact.*, 1944, **47**, 26. The present author wishes to express his appreciation for permission to use this medium before full details of its composition have been published.

2 Ward, H. K., and Rudd, G. V., *Australian J. Exp. Biol. and M. Sc.*, 1938, **16**, 181.

† Glutamine should be kept in a desiccator over calcium chloride in the refrigerator at 4°C.

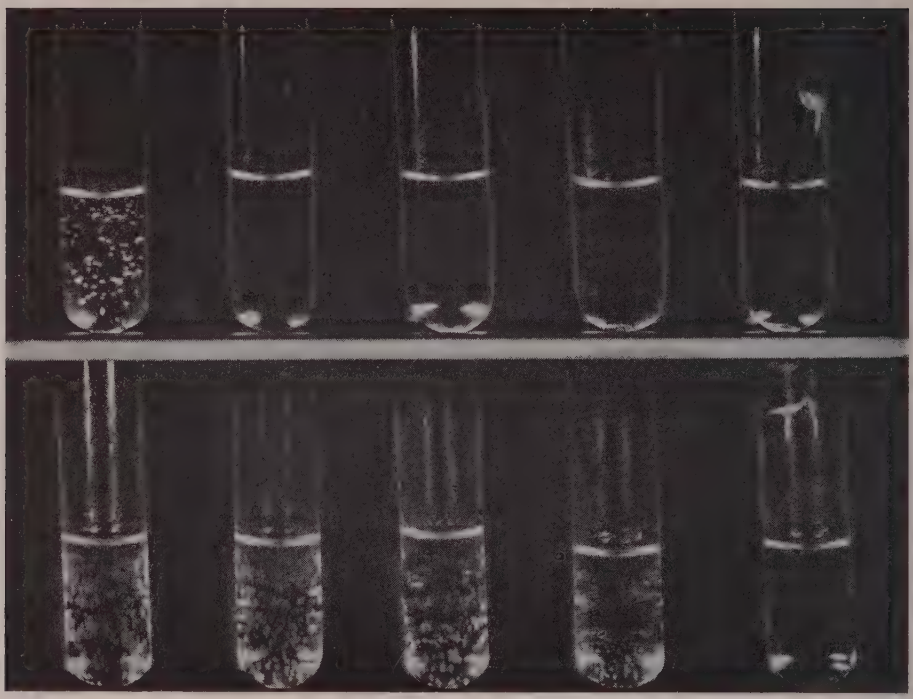

FIG. 1.
Sulfonamide Resistance Tests.
Top: A fully susceptible strain. Result "0." Bottom: A highly resistant strain. Result "25." The tubes from left to right contain respectively 0, 1, 5, 25, and 125 mg of sodium sulfadiazine per 100 cc of medium.

minutes.

Solutions VI R and VI H must be prepared immediately before use. All of the others can be made in advance and stored in the ice box.

Technic. To perform the test, sets of the various solutions are mixed in each of 5 sterile, suitably sized Erlenmeyer flasks according to the appended dilution schedule (or in fractions or multiples thereof, depending on the number of strains to be tested). Solutions I, V, VI R and rabbit serum, or VI H and horse serum, saline and sodium sulfadiazine are mixed in the flasks which are then placed in a 48°C water bath. Solution VII is melted in the autoclave and is placed in the same water bath. When all solutions have reached the proper temperature, the indicated quantity of Solution VII is added to each flask by means of warmed pipettes; and the final solutions are then distributed in 2.0 cc amounts in sterile 13 x 100 mm test tubes. Thus 5 tubes contain the test media for a single strain and each contains respectively a concentration of 0, 1, 5, 25, and 125 mg of sodium sulfadiazine per 100 cc of the material.

Strains to be tested are grown overnight in 2.0 cc of medium composed of 80% of Solution I, 5% of Solution V, and either 5% of Solution VI R plus 10% of rabbit serum or 5% of Solution VI H and 10% of horse serum, according to the serum to be employed the following day for the final test.

A small loopful (the loop is made by winding 26-gauge platinum wire around an 18-gauge needle) of this overnight culture is transferred to 2.0 cc of Solution I in a test tube. This dilute cell suspension is agitated sharply 30 times, and a loopful of it is transferred to each of the 5 tubes constituting the test. The loop is flamed between inoculations. The inoculum is considered to be of satisfactory size if 10 to 100 colonies grow in the tube

which contains no sulfonamide. If the number of colonies is not within these limits the test must be repeated with a more suitable inoculum.

The tubes are incubated at 37°C for 18 to 24 hours; and the test is read in terms of the concentration of sodium sulfadiazine in the highest tube in which visible growth occurs. If this be in the control tube only the result is "0"; if in all tubes except that containing 125 mg of the drug per 100 cc of medium the result is "25." Tests showing values of 0 and 25 are shown in Fig. 1.

Discussion. Results have been reproducible with a variation of plus or minus one tube. Strains have been kept on blood agar slants in the ice box for at least one month without undergoing a change in their *in vitro* resistance to sulfadiazine.

To obtain consistent results the same sulfonamide and serum from the same species must be used. Sodium sulfathiazole has given readings about one tube lower than sodium sulfadiazine; and medium made with rabbit serum has given readings about one tube higher than that made with horse serum and xanthine.

The semi-synthetic part of the medium is doubtless unnecessarily complex, because several of its constituents are contributed in sufficient quantity by the added serum. No attempt has, as yet, been made to eliminate these superfluous materials or to reduce the medium to its simplest form.

Many strains of hemolytic streptococci are inhibited by as little as 0.2 mg of sodium sulfadiazine per 100 cc of the medium, which indicates that the content of substances antagonistic to sulfonamide action is very low.

No success has attended attempts to refine the test in such a manner that lesser degrees of sulfonamide resistance might be demonstrated. The intervals between the concentrations of drug in the tubes (multiples of 5) have been chosen deliberately; and the good correlation which exists between the findings of the test and clinical observations in the field[3] suggests that it is sufficiently precise to be of practical value.

Summary. A technic for testing the resistance of Group A hemolytic streptococci to sulfonamides is presented. The test has been found to be sufficiently precise to indicate clinically significant differences in sulfonamide susceptibility.

[3] Wilson, A. T., and Coburn, A. F., to be published.

14871

Competition of Antigens in Isoimmunization by Pregnancy.*

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When an experimental animal is injected with a mixture of two substances, one a good antigen and the other a poor antigen, the antigenicity of the weaker antigen is often suppressed.[1] The purpose of the present communication is to report some observations in man of this phenomenon, known as competition of antigens.

The properties A and B are good antigens in man, as revealed by observations made following inadvertent transfusions of blood of an incompatible group.[2] On the other hand, only 1 in 25 to 50 Rh-negative individuals respond to transfusions of Rh-positive blood, or pregnancy with an Rh-positive fetus, by

* Aided by a grant from the United Hospital Fund of New York City.

[1] *Cf.* Landsteiner, K., *The Specificity of Serological Reactions*, 2d edition, p. 104, Harvard Univ. Press, Boston, 1945.

[2] Wiener, A. S., *Blood Groups and Transfusions*, p. 123, 3rd ed., C. C. Thomas, Springfield, Ill., 1943.

TABLE I.
Comparison of the Incidence of Compatible Matings in Families with Erythroblastotic Infants, and Their Incidence in Normal Families.

Families with erythroblastotic infants				Control, normal families				
Investigators	Total No. of families	Compatible % ± P.E.	Incompatible %	Investigators	Total No. of families	Compatible % ± P.E.	Incompatible %	Difference % ± P.E.
Present study	96	71.9 ± 3.0	28.1	Wiener *et al.*§	215	63.2 ± 2.2	36.8	+ 8.7 ± 3.7
Levine*	215	75 ± 1.9	25	Calculated from distribution of groups in N. Y. C.	—	65	35	+10 ± 1.9
Race, Taylor, Cappell and McFarlane†	40	70.0 ± 3.5	30.0	Taylor, Race *et al.*¶	236	64.4 ± 3.1	35.6	+ 5.6 ± 4.7
Broman‡	33	81.8 ± 4.5	18.2	Calculated from distribution of groups in Sweden	—	64.2	35.8	+17.6 ± 4.5

* *J. Hered.*, 1943, **34**, 71.
† *Brit. Med. J.*, 1943, **2**, 289.
‡ *Acta Paed.*, 1944, Vol. XXXII, Suppl. II.
§ PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 167; *Genetics*, 1943, **28**, 157; *J. Exp. Med.*, 1944, **79**, 235.
¶ *Ann. Eug.*, 1938, **8**, 343; *ibid.*, 1942, **11**, 385.

producing Rh antibodies.[3,4,5] Therefore, for example, if a group O Rh-negative woman bearing a group A Rh-positive fetus becomes isoimmunized, it would be expected that she would either produce antibodies for A alone, or antibodies for A and Rh simultaneously, but not for Rh alone. In general, moreover, it would be expected that an Rh-negative woman is more apt to become sensitized to the Rh factor present in her fetus if the fetus belongs to a compatible blood group than if the fetus' group is incompatible. That this is actually the case can be seen from Table I, in which the incidence of compatible matings in families with erythroblastotic infants is compared with the incidence of compatible matings in a random series of families without children having hemolytic diseases. (The excess of compatible matings in families with erythroblastotic infants has also been noticed by Levine,[6] but no attempt was made to explain it.) In cases in which an Rh-negative woman had an erythroblastotic baby, who belonged to an incompatible blood group, and titration experiments were carried out, the present author has usually found, in accordance with expectation, a high titer of the corresponding specific isoagglutinin in the maternal serum.

These observations can be applied in cases of stillbirths or neonatal deaths of obscure etiology, in which the mother is Rh-negative, the father and fetus (or infant) Rh-positive, but the maternal serum does not contain any detectable Rh agglutinating or blocking antibodies.[5] In the absence of such isoantibodies, the possibility still remains open that the mother may not be sensitive to the Rh factor and the fact that she is Rh-negative may merely be a coincidence. This interpretation is supported if the fetus (or infant) belongs to an incompatible blood group, and the corresponding isoagglutinin in the maternal serum is nevertheless of only average titer. On the contrary, the diagnosis of erythroblastosis is rendered more probable if the corresponding

3 Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

4 Wiener, A. S., *Arch. Path.*, 1941, **32**, 227.

5 Wiener, A. S., Wexler, I. B., and Gamrin, E. L., *Am. J. Dis. Child.*, 1944, **68**, 317.

6 Levine, P., *J. Hered.*, 1943, **34**, 71.

specific isoagglutinin in the maternal serum is of high titer or if the fetus (or infant) belongs to a compatible blood group. Determining the titer of the maternal isoagglutinins is also of value in the less common cases of suspected hemolytic disease in which the mother is Rh-positive, because here one must still consider the possibility of isoimmunization against factor Hr, or the isoimmunization of an individual of one Rh type against blood of a different Rh type.

The phenomenon of competition of antigens also manifests itself with regard to the different varieties of Rh factors.[7] Of the 3 Rh factors, Rh_o is by far the most antigenic, Rh′ is less antigenic, and Rh″ is the least antigenic.[8] Therefore, it would be expected that if, for example, an Rh-negative woman bears a type Rh_1 fetus and becomes sensitized to the Rh factor, her serum should either contain anti-Rh_o alone, or anti-Rh_o and anti-Rh′ together, but not anti-Rh′ alone. Actual observations fully satisfy these expectations; the cases where the maternal sera appeared to contain anti-Rh′ alone all proved actually to be examples of anti-Rh'_o sera with anti-Rh_o blocking antibodies.[9,10,11] It is highly significant that even though a high percentage of erythroblastotic infants from Rh-negative mothers belong to type Rh_2, only two instances of anti-Rh''_o sera from such mothers have been encountered[12] and none of anti-Rh″ alone, in contrast to the numerous anti-Rh_o sera such cases have yielded. On the other hand, in the far rarer instances of the type Rh_1 mother bearing an erythroblastotic infant of type Rh_2 or type Rh_1Rh_2, as many as 7 anti-Rh″ sera have been observed already, two by the writer[13] and five by British investigators.[14]

These observations on competition of antigens in man may possibly find application in the prophylaxis of hemolytic disease of the fetus and newborn. While specific desensitization of the mother by injections of purified Rh haptens has not proved practicable to date, it is possible that counter-immunization of the mother during pregnancy with a potent but innocuous vaccine may serve to suppress the formation of Rh isoantibodies, and in that way prevent or diminish the severity of the disease in the fetus.

7 For nomenclature, see Wiener, A. S., *Science*, 1944, **99**, 532.

8 Wiener, A. S., *Am. J. Clin. Path.*, in press.

9 Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 173.

10 Race, R. R., *Nature*, 1944, **153**, 771.

11 Wiener, A. S., Davidsohn, I., and Potter, E. L., *J. Exp. Med.*, 1945, **81**, 63.

12 Wiener, A. S., and Sonn, E. B., *J. Immunol.*, 1943, **47**, 461; Wiener, A. S., unpublished observations.

13 Wiener, A. S., unpublished observations.

14 Loutit, J. F., personal communication.

14872

Pituitary-Adrenal Cortical Control of Antibody Release from Lymphocytes. An Explanation of the Anamnestic Response.*

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The lymphocytes of normal rabbits contain a globulin identical with the normal serum gamma globulin of the rabbit.[1] Labeled globulin, *i.e.*, antibody protein, was demonstrated in lymphocytes obtained from lymphoid tissue of immunized mice.[2] The

* This investigation has been aided by grants from the Josiah Macy, Jr., Foundation, and the Fluid Research Fund of Yale University School of Medicine.

† Alexander Brown Coxe Memorial Fellow.

1 White, A., and Dougherty, T. F., *Abs. Proc. Meetings of Am. Chem. Soc., New York, September*, 1944; *Endocrinology*, in press.

presence of immune bodies in lymphocytes has recently been confirmed in another species, the immunized rabbit.[3] Further, it has been demonstrated that augmented pituitary-adrenal cortical secretion accelerates the rate of release of antibodies from the lymphoid tissue of immunized rabbits.[4] The mechanism of this release is the marked dissolution of lymphocytes which occurs in lymphoid structures within a few hours after increased adrenal cortical secretion.[5] Histological changes in the lymphoid tissue, characterized by lymphocyte dissolution, are reflected in the marked absolute lymphopenia which occurs concomitantly.[6]

Further proof of the role of lymphocytes as a storehouse of gamma globulin and of the hypothesis that pituitary-adrenal cortical secretion controls the rate of release of this globulin, was sought in studies with immunized animals whose sera contained no demonstrable antibodies. The administration of adrenotrophic hormone or of adrenal cortical extracts would be expected to release antibody into the blood at a time when lymphocyte dissolution and lymphopenia are most marked. Moreover, the recognition that a wide variety of unrelated stimuli known to augment pituitary-adrenal cortical secretion[7] are also effective in producing enhancement of antibody titers in the sera of previously immunized animals[8] suggested an explanation of the mechanism of the anamnestic reaction. The enhancement of antibody titer which occurs following the injection of a variety of non-specific substances other than the original antigen has been called the anamnestic reaction.

Experimental. Rabbits and mice were used in this study. The rabbits were males of mixed parentage, approximately 4 months old at the start of the experiments. The mice were of both sexes (NHO strain, Strong) and were 60 to 80 days old at the time the antigen was first injected. The rabbits received commercial chow pellets and oats, and the mice were fed Purina Fox Chow supplemented with calf meal. Food and water were available at all times.

A group of 12 rabbits was injected intravenously on alternate days with 2 ml of a 5% suspension of washed sheep erythrocytes. Agglutinin titers were estimated at intervals on ear vein blood. At the end of 9 weeks the maximum titers obtained were approximately 1-5000. The rabbits then remained in the laboratory for 3 months; at the end of this time no sheep cell antibodies were demonstrable in the serum.

A group of mice was immunized by intraperitoneal injection of 1 ml of a 2% suspension of washed sheep erythrocytes 3 times weekly for 14 weeks. At this time agglutinin titers done on the pooled sera (heart blood) of several groups of 5 animals were 1 to 700. Antigen injection was discontinued and at the end of 4 weeks similar agglutinin titers on 5 groups of 5 mice each showed no circulating sheep cell antibody.

Twelve rabbits and 90 mice prepared in the above manner were used in subsequent experiments. Adrenalectomized mice were used 16 hours after operation. Two types of adrenal cortical extracts were employed, an aqueous preparation (Wilson) and a solution of adrenal cortical steroids in oil kindly supplied by Dr. E. Gifford Upjohn of the Upjohn Company. The desoxycorticosterone acetate was a product of the Schering Corporation. Purified pituitary adrenotrophic hormone (adrenotrophin) was prepared from hog pituitary glands.[9] Potassium arsenite and thiophene-free benzene were commercial products of established purity.

Lymphocyte extracts were prepared as previously described.[2] Agglutinin titers were usually done on lymphocyte extracts and sera using the doubling dilution method. Addi-

[2] Dougherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 295.

[3] Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.*, 1945, **81**, 73.

[4] Dougherty, T. F., White, A., and Chase, J. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 28.

[5] White, A., and Dougherty, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 26.

[6] Dougherty, T. F., and White, A., *Endocrinology*, 1944, **35**, 1.

[7] Sayers, G., Sayers, M. A., Fry, E. G., White, A., and Long, C. N. H., *Yale J. Biol. and Med.*, 1944, **16**, 361.

[8] Cannon, P. R., *J. Immunol.*, 1942, **44**, 107.

[9] Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, 1943, **149**, 425.

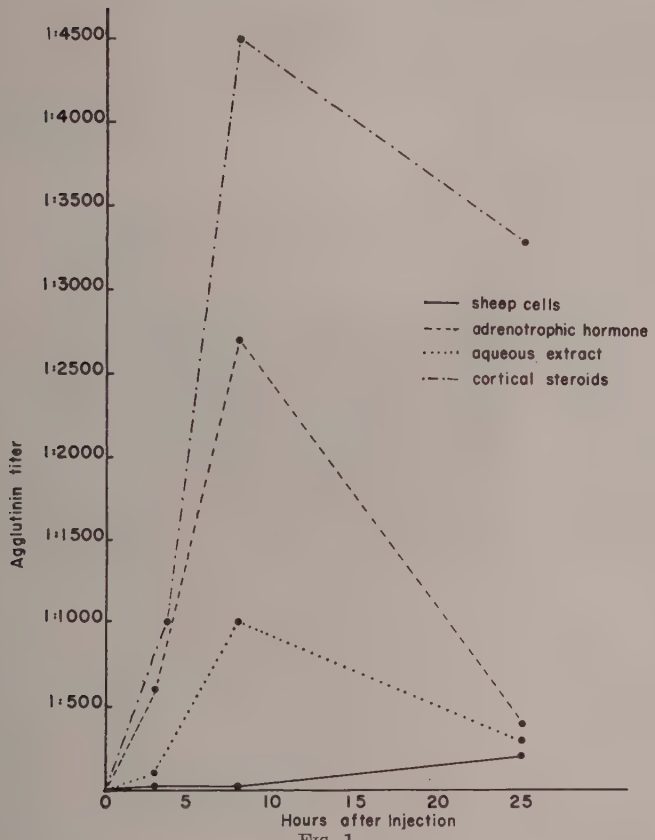

FIG. 1.

Anamnestic response in rabbits. Each curve is the average data for a group of 3 rabbits.

tional dilutions of rabbits' sera were titrated yielding data on dilutions between the intervals of the doubling dilution technic. In the studies with mice each experiment represents the pooled sera and aggregate lymphoid tissue of 5 animals.

Results. The rabbit data which have been obtained are shown in Fig. 1. A single, subcutaneous injection of 5 ml of the oil solution of adrenal cortical steroids produced demonstrable serum agglutinins at 3, 9, and 25 hours after hormone administration (Fig. 1). These were the only intervals at which the bloods were examined. The titers at the 9-hour interval were approximately as high as those which had been reached at the time antigen administration had been discontinued, *i.e.*, maximum. The injection of a single, subcutaneous dose of 10 mg of adrenotrophic hormone in 2 ml water or of 10 ml aqueous adrenal cortical extract also produced a marked release of antibody with the maximal effect evident in the blood at the 9-hour period. In contrast to the definite effect of the 3 types of hormone preparations, a single, intravenous injection of 10 ml of the 5% erythrocyte suspension used as the original antigen produced only a slight anamnestic response. Unpublished results have shown that intravenous injection of sheep cells in the normal rabbit gives evidence of pituitary-adrenal cortical stimulation as manifested by an absolute lymphopenia 3 to 6 hours after the injection.

The data obtained in the experiments with the mice are depicted in Fig. 2. Within 3 hours after the subcutaneous injection of 0.5 ml of aqueous adrenal cortical extract, the titer rose from 0 to 1 to 640 in the non-operated

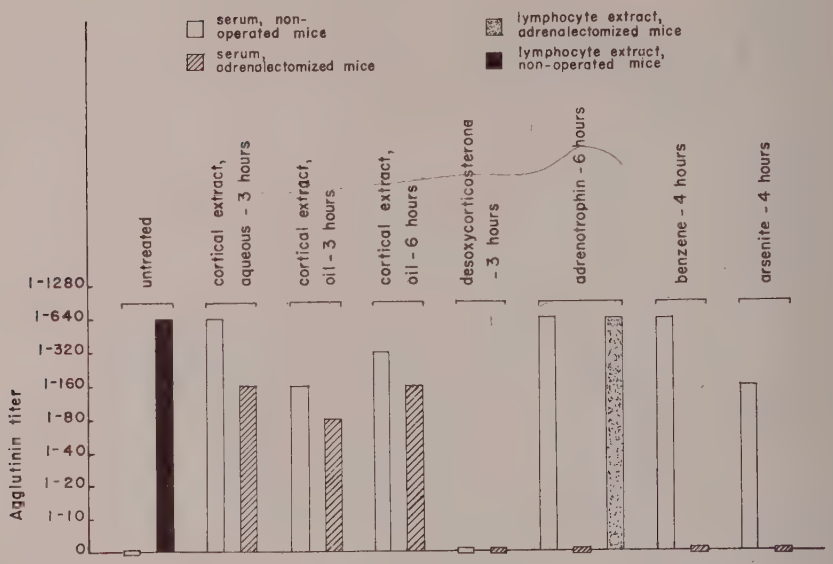

FIG. 2.
Anamnestic response in mice.

animals and from 0 to 1 to 160 in the adrenalectomized group. Within 6 hours after the subcutaneous injection of adrenal cortical steroids in oil, 0.25 ml in the non-operated animals and 0.5 ml in the adrenalectomized mice, antibody titers of 1 to 320 were found in the former and 1 to 160 in the latter. Desoxycorticosterone acetate (1.25 mg in 0.25 ml of oil) was injected subcutaneously into each of 5 non-operated and 5 adrenalectomized mice. No demonstrable antibodies were detected in the pooled sera of either of these groups within 3 hours following the injection. In another group of mice, the subcutaneous administration of adrenotrophic hormone (1 mg in 0.5 ml water) into each mouse produced a titer of 1 to 640 in the non-operated mice within 6 hours. Similar injections of adrenotrophic hormone into adrenalectomized mice did not induce an anamnestic response. Extracts of lymphocytes of these same adrenalectomized mice, treated with adrenotrophic hormone, showed an agglutinin titer of 1 to 640.

Two groups of animals receiving toxic chemical agents were used in order to ascertain whether an anamnestic response could be induced. These substances, potassium arsenite and benzene, have previously been shown to produce adrenal cortical activation, lymphocyte dissolution and lymphopenia in mice.[10] The lymphocyte changes occurred in normal but not in adrenalectomized mice. It may be seen from Fig. 2 that the subcutaneous injection of 0.03 ml of benzene or of 0.1 mg of potassium arsenite (in 0.25 ml water) produced a marked anamnestic reaction in the non-operated animals while none occurred in the adrenalectomized mice.

It should be emphasized that of the animals represented by the data in Fig. 1 and 2 no rabbit nor any group of mice failed to exhibit the anamnestic response as depicted. Data substantiating those in Fig. 1 have been obtained in another group of rabbits in the course of other experiments.

Discussion. The anamnestic response is dependent upon the release of antibody from lymphocytes as a result of pituitary-adrenal cortical stimulation. The presence of gamma globulin in the lymphocytes of normal animals[1] and of labeled globulin in lymphocytes of immunized animals[2] has been demonstrated pre-

[10] Dougherty, T. F., and White, A., unpublished results.

viously. The control of lymphoid tissue size[11,12] and of the numbers of circulating lymphocytes[6] by adrenal cortical hormones is primarily due to the influence of these hormones upon the dissolution of lymphocytes *per se*.[5] Since the dissolution of lymphocytes occurs within a 3- to 6-hour interval following injection of adrenal cortical hormones or of adrenotrophic hormone, the time interval at which antibody globulin appears in the blood following hormone injection should coincide with the retrogressive changes in lymphocytes. This coincidence has been previously demonstrated in hyperimmunized animals.[4] The anamnestic response occurring within 3 to 9 hours in rabbits and mice following a single injection of either aqueous adrenal cortical extract, adrenal cortical steroids in oil, or adrenotrophic hormone further substantiates the relationship between lymphocyte dissolution and globulin contribution to the serum.

The mediation of the effect of the pituitary adrenotrophic hormone by way of the adrenal cortex is seen from the fact that the adrenotrophic hormone elicited no anamnestic response in adrenalectomized mice. However, the lymphocytes of these same mice contained appreciable quantities of antibody. The presence of adrenal cortical steroids is therefore essential for the liberation of antibody globulin from lymphocytes.

The failure of desoxycorticosterone acetate to elicit a response in either the non-operated or the adrenalectomized mice confirms the inability of this steroid to affect lymphoid tissue histology,[10] level of blood lymphocytes,[6] or gluconeogenesis.[1,6,13]

The possibility that antibody might be adsorbed on lymphocytes has been previously considered unlikely.[2] Other investigators have also recognized this possibility.[3] The present data confirm our previous conclusions that antibodies are an integral part of the lymphocyte rather than being adsorbed on the cell from the surrounding medium. Lymphocytes containing considerable quantities of labeled globulin have been obtained from animals with no circulating antibody. It is obvious that since the serum contained no antibody protein, adsorption or absorption of antibody by the lymphocytes could not have occurred. The presence of immune bodies within lymphocytes is not interpreted as evidence that lymphocytes are necessarily concerned with antibody formation.

Many types of stimuli may induce the anamnestic reaction, *e.g.*, non-specific protein injections, hemorrhage, hyperthemia, toxic chemicals, etc. Some of these have been demonstrated to produce pituitary-adrenal cortical activation resulting in lymphoid tissue dissolution, lymphopenia, and an increase in total serum proteins.[10] Of these stimuli which have been studied, benzene and arsenite were very effective in producing the above mentioned alterations in normal mice but to be totally ineffective in adrenalectomized or hypophysectomized mice. Therefore, these two toxic agents were examined in the present study and showed an anamnestic effect in the non-operated mice but not in the adrenalectomized animals. The anamnestic reaction must be based upon the release of antibodies due to the effect of the adrenal cortical steroids on lymphocytes. The data presented further integrate the role of the lymphocyte and of the adrenal cortex in the normal defense mechanisms of the organism.

Summary. An anamnestic reaction has been produced in rabbits and mice following a single injection of adrenal cortical extract or pituitary adrenotrophic hormone. Desoxycorticosterone acetate injection failed to elicit this response. In adrenalectomized mice the anamnestic reaction also was elicited by adrenal cortical extracts but not by adrenotrophic hormone, despite the demonstrated presence of antibodies in the lymphocytes of these animals. Therefore, adrenal cortical mediation is essential for control of the release of antibody from lymphocytes.

Two toxic stimuli, benzene and potassium arsenite, liberated antibodies from lymphocytes in intact mice. These stimuli failed to effect this release in adrenalectomized mice. The data establish the role of pituitary-adrenal

[11] Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

[12] Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., *ibid.*, 1943, **54**, 135.

[13] Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinology*, 1940, **26**, 309.

cortical secretion as the controlling mechanism for the release of antibody from lymphocytes. The anamnestic reaction is one manifestation of this control.

14873

Pyridoxine and Tryptophane Metabolism in Rice Moth Larvæ (*Corcyra cephalonica St.*).

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From the Nutrition Research Laboratories, Indian Research Fund Association, Coonoor, S. India.

Lepkovsky and Nielsen[1] showed that the urine of rats which were kept on a pyridoxine-deficient diet contained a yellow pigment, which turned green at neutral pH on the addition of ferric salts like ferric ammonium sulphate. Later, Lepkovsky, Roboz, and Haagen-Smit[2] isolated the yellow pigment by chromatographic adsorption procedure and identified it as xanthurenic acid. It disappeared from the urine of rats when they were transferred from the deficient diet to one rich in pyridoxine or when pyridoxine was given orally. This compound was first studied by Musajo,[3] who found it in the urine of rats and rabbits fed high protein diets.

It has been shown by the author[4] that pyridoxine is one of the growth promoting factors for the rice moth larvæ (*Corcyra cephalonica* St. Lep.). It was therefore of interest to determine whether the rice moth larvæ excreted any yellow colored compounds in the feces when they were kept on a pyridoxine-deficient diet containing added amounts of tryptophane.

The pyridoxine-deficient diet consisted of salt-extracted wheat flour, sugar, and salt mixture together with the vitamins thiamine, riboflavin, nicotinic acid, and calcium pantothenate in the proportion of 10, 5, 50, and 15 γ per g of diet respectively. Rice moth larvæ which were feeding on whole wheat for a period of 10-12 days after hatching were removed, cleaned, weighed, and placed on the diets given in Table I. The color of the excreta was noted after one week.

These results indicate that on a pyridoxine-deficient diet containing additional amounts of tryptophane, the larvæ excreted yellow-colored feces. Further, it was found that the yellow color disappeared from the feces of the larvæ when the latter were transferred to diets rich in pyridoxine. The same changes in the color of the larval excreta were observed even when bigger larvæ, *i.e.*, larvæ which had fed on whole wheat for 15-20 days after hatching, were placed on the diets mentioned in Table I.

The following experiments were carried out to determine whether the incorporation of tryptophane in the various deficient diets caused the yellow-colored excreta. Rice moth larvæ were taken from whole wheat, divided into 6 equal batches of 20 larvæ and placed on the diets given in Table II.

The results clearly show that only those larvæ which were placed on a diet deficient in pyridoxine excreted yellow-colored feces.

Seven amino acids, cystine, tryptophane, lysine, monohydrochloride, histidine, *l*-leucine, *d*-arginine, and tryosine were fed to the rice moth larvæ at a level of 50 mg of the amino acid to every 5 g of the pyridoxine-deficient diet. It was found that only tryptophane caused the excretion of the yellow-colored feces.

These results clearly indicate that there is an intimate relationship between pyridoxine and tryptophane metabolism in the rice moth larvæ.

The minimum amount of tryptophane

[1] Lepkovsky, S., and Nielsen, E., *J. Biol. Chem.*, 1942, **144**, 135.

[2] Lepkovsky, S., Roboz, E., and Haagen-Smit, A. J., *Ibid.*, 1943, **149**, 195.

[3] Musajo, L., *Chem. Abst.*, 1935, **29**, 6292; *Boll. Soc. ital. biol. sper.*, 1935, **10**, 290.

[4] Sarma, P. S., *Ind. J. Med. Res.*, 1943, **31**, 165.

TABLE I.

Diet	Color of the larval excreta	Wt in mg for 10 larvæ: Initial	Wt in mg for 10 larvæ: After 2 weeks
1. Pyridoxine-deficient diet + 10 mg tryptophane per g diet	yellow	4.40	12.20
2. Pyridoxine-deficient diet + 5 mg tryptophane per g diet	yellow	4.35	13.24
3. Basal diet + all vitamins + 5 mg tryptophane per g diet	white	4.61	59.32
4. Basal diet + all vitamins	white	4.50	61.15
5. Pyridoxine-deficient diet	white	4.44	13.12

TABLE II.
Weight of Larvæ and Color of Excreta on Different Diets.

Diet	Color of the excreta	Wt in mg for 10 larvæ: Initial	Wt in mg for 10 larvæ: After 2 weeks
1. 5 g riboflavin-deficient diet + 50 mg tryptophane	white	3.60	8.80
2. 5 g nicotinic acid-deficient diet + 50 mg tryptophane	"	3.70	37.23
3. 5 g basal diet + all vitamins + 50 mg tryptophane	"	3.50	38.91
4. 5 g pyridoxine-deficient diet + 50 mg tryptophane	yellow	3.50	8.22
5. 5 g pyridoxine-deficient diet + 100 mg tryptophane	"	3.72	9.56
6. Basal diet + all vitamins	white	3.75	36.63

which had to be added to the pyridoxine-deficient diet to produce a distinctly yellow-colored larval excreta was found to be about 3 mg per g of the diet.

The yellow pigment was found to be readily soluble in water, slightly soluble in aqueous acetone and aqueous alcohol, but insoluble in ether, absolute alcohol, benzene, chloroform, and acetone. The addition of mercuric sulphate solution (15%) caused its precipitation, as a mercury complex compound, but it was found difficult to purify the pigment and concentrate it, since hydrogen sulphide, which was used to remove the mercury, destroyed it.

However, the yellow pigment could be adsorbed on fuller's earth (B.D.H.) in acid medium and could also be easily eluted by treatment with dilute alkali solution. A concentrated solution was prepared in this manner, but attempts at obtaining the pigment in a pure crystalline form proved unsuccessful.

In aqueous solution the yellow pigment did not turn green on the addition of ferric ammonium sulphate at neutral pH. Thus it does not show the most characteristic reaction of xanthurenic acid. Probably we are dealing with a pigment entirely different from xanthurenic acid and further investigation of its occurrence in larval excreta may throw more light on pyridoxine and tryptophane metabolism.

Summary. A yellow-colored compound is excreted by rice moth larvæ, when they were fed pyridoxine-deficient diets containing tryptophane. The yellow color disappeared from the excreta either on the inclusion of pyridoxine in the diet or on the transference of the larvæ to a pyridoxine-rich diet. Of the 7 amino acids examined, only tryptophane gave rise to the yellow-colored excreta. The pigment is soluble in water, adsorbed on fuller's earth, but does not turn green with ferric ammonium sulphate at neutral pH. The compound in question may, therefore, be different from xanthurenic acid.

14874 P

Growth Stimulation of Neurospora Cholineless Mutant by Dimethylaminoethanol.

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It was recently noted[1] that dimethylaminoethanol had a choline-like effect in promoting growth and preventing perosis in chicks, and it was thought that this might be due to the addition of a "labile" methyl group to dimethylaminoethanol to form choline. It has been observed that the chick,[2] in contrast to the rat, is unable to use methionine or betaine as a substitute for choline when fed with purified diets. The "cholineless" mutant of *Neurospora crassa* No. 34486 appears to resemble the chick rather than the rat, for this mutant is unable to use betaine as a substitute for choline, and methionine has very little effect.[3] It was thought that a combination of betaine or methionine with dimethylamino-

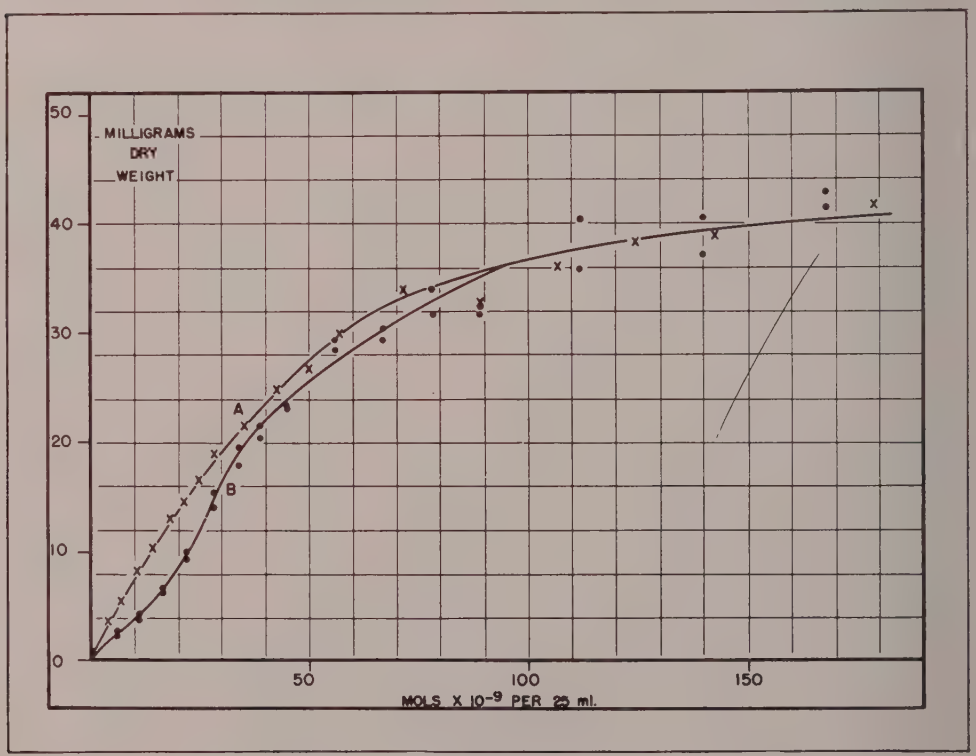

Fig. 1.

Growth of cholineless mutant No. 34486 on choline chloride (curve A) and dimethylaminoethanol (curve B) after 3 days. Choline chloride = ×; dimethylaminoethanol = ●.

[1] Jukes, T. H., and Oleson, J. J., *J. Biol. Chem.*, 1945, **157**, 419.

[2] Jukes, T. H., *J. Nutrition*, 1941, **22**, 315; Almquist, W. J., and Grau, C. R., *Ibid.*, 1944, **27**, 263; McGinnis, J., Norris, L. C., and Heuser, G. F., Proc. Soc. Exp. Biol. and Med., 1944, **56**, 197.

[3] Horowitz, N. H., and Beadle, C. W., *J. Biol. Chem.*, 1943, **150**, 325.

ethanol might stimulate the growth of the mutant.

The assay with the "cholineless" mutant* was carried out by the method of Horowitz and Beadle.[3] Aminoethanol and betaine were inactive and methionine had a very slight activity, which confirms a previous report.[3] It was found that dimethylaminoethanol alone or in combination with betaine or methionine had a marked effect in stimulating growth. A comparative assay showed that dimethylaminoethanol alone was approximately 1.5 times as active as choline chloride. When calculated to a molar basis this result indicates that the two compounds show about the same activity in the upper part of the response curve, but in the lower portion of the curve the response to choline was greater than the response to dimethylaminoethanol. This is illustrated in Fig. 1. Diethylaminoethanol was found to have less than 0.1% of the activity of dimethylaminoethanol.

A solution of dimethylaminoethanol, 0.5 mg per cc, was submitted to permutit adsorption and elution[3] and the eluate and filtrate were assayed with the mutant. The biological activity was found to pass quantitatively into the eluate. The filtrate was inactive.

A solution of choline chloride was submitted to reineckate precipitation.[4] The reineckate precipitate was measured colorimetrically and the filtrate was assayed with the mutant. When dimethylaminoethanol was added to the choline chloride solution, the dimethylaminoethanol appeared to be partially precipitated, as indicated by the increased colorimetric value of the precipitate and the increased assay value of the filtrate. Dimethylaminoethanol in pure solution, however, was not precipitated with reineckate under the conditions used.

The results with the mutant might indicate that it is able to synthesize a "methyl donator," such as, perhaps, methionine, but is defective in being unable to synthesize the postulated "methyl acceptor," dimethylaminoethanol, in the formation of choline.

* Cultures of the mutant were kindly supplied by Dr. N. H. Horowitz and by Dr. Earle Arnow.

4 Glick, D., *J. Biol. Chem.*, 1944, **156**, 643.

14875

A Method for Production of a Localized Gonococcal Infection in the Rabbit's Eye.*

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Investigation of certain problems related to gonococcal infection have been neglected because of lack of a satisfactory method of producing a localized infection experimentally in some laboratory animal. Among the many attempts reported in the literature[1] only that by Cohn[2] warrants mention here. He injected gonococci into the anterior chamber of rabbits' eyes and occasionally succeeded in recovering them from that site several days thereafter and on 2 occasions 28 days after inoculation with 2 particularly virulent strains. He, nevertheless, concluded that the longevity of the microörganisms was "variable and irregular" and that the anterior chamber of the rabbit was unsuitable for his purposes.

The method described below was developed

* The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

1 For a comprehensive bibliography see the excellent review of the literature by Justina Hill, *Am. J. Syph., Gon. and Ven. Dis.*, 1944, **28**, 334 and 471.

2 Cohn, Alfred, *Derm. Z.*, 1930, **60**, 35.

after a protracted search for an experimental infection which would lend itself to the testing of gonococcidal agents by topical application *in vivo* such as is impractical in the mouse infection previously described.[3,4]

Materials. Animals: Young adult rabbits were used for most experiments. They were easier to handle than very young rabbits and seemed to be no less susceptible to infection. Albinos possessed two advantages—the progress of the inflammatory reaction could be more easily observed and smears of the ciliary body contained no pigment.

Strains of Gonococci Employed: Two strains of gonococci were used: one had been isolated in 1942 from the blood of a patient with bacterial endocarditis and maintained in mice by continuous animal passage.[5] At the time of its 133rd mouse passage it was found to be satisfactory for infection of the anterior chamber of the rabbit's eye. The other strain was freshly isolated from the exudate of a gonococcal urethritis which had resisted sulfathiazole for many weeks.

The medium was made by digesting a 10% suspension of casein with trypsin, boiling, filtering, diluting with 3 parts of water and adding the following ingredients: 0.65% $Na_2HPO_4 \cdot 12H_2O$, 0.50% KCl, 1.00% dextrose, 1.80% agar, 0.065% cystine, heating and adjusting to pH 7.8 which should come down during sterilization to approximately 7.4. For the recovery of gonococci from infected eyes, this medium was enriched by the addition of about 10% defibrinated rabbit blood.

Methods. Inoculation: Cultures to be used for inoculation were grown over night on the casein digest-cystine agar described above. A loopful of culture was suspended in a few ml of saline and diluted to a density which experience has shown to contain approximately 1,000,000,000 gonococci per ml. Maximal dispersion of the microörganisms was insured by repeated sucking in and out of the pipette.

Other suspending fluids were tried; *e.g.*, water, broth, gelatin, Locke's solution and mucin in concentrations of 2 and 4%,† but none possessed any advantage over saline. Mucin increased the inflammatory reaction to an undesirable degree.

The rabbits were prepared for inoculation by intravenous injection of 30 mg of morphine sulfate in 1 ml of saline and instillation of a drop of 5% cocaine into each conjunctival sac. The hair around the lid margins including lashes and whiskers was clipped to eliminate a source of contamination. The lids were opened wide and held in that position by a speculum. A fine hypodermic needle attached to a 2 cc Luer syringe was inserted at the limbus into the anterior chamber and the aqueous humor aspirated by gentle suction. This needle was withdrawn and another of the same caliber attached to a 1 ml tuberculin syringe containing a suspension of gonococci inserted through the same hole and 0.2 ml of bacterial suspension injected into the anterior chamber.

After inoculation the rabbits were kept in a room which was artificially cooled during the hot weather to a temperature between 50° and 60° F.

Recovery of gonococci from infected eyes: When aqueous humor was to be withdrawn for culture or transfer before the animals were to be sacrificed, they were prepared by general and local anesthesia as described above and the aqueous was withdrawn into a 1 or 2 ml syringe. When complete cultures were required the animal was sacrificed by an intravenous injection of air, the aqueous aspirated and cultured by spreading two drops over the surface of a freshly prepared blood-agar plate. The eye was then enucleated, rinsed in 70% alcohol, and opened by cutting the cornea with a sharp knife. A portion of the ciliary body was removed with sterile forceps, macerated and streaked over the surface of another blood agar plate. The lens was then removed and its anterior surface cultured in the same way. The vitreous humor was cultured by streaking

[3] Miller, C. Phillip, "Experimental Gonococcal Infection," in *The Gonococcus and Gonococcal Infection*, Publication No. 11, A.A.A.S., Lancaster, Pa., 1939, 20.

[4] Miller, C. Phillip, and Hawk, Walter D., *Trans. Assn. Am. Phys.*, 1940, **55**, 216.

[5] Miller, C. Phillip, *Am. J. Syph., Gon. and Ven. Dis.*, 1944, **28**, 620.

† Granular mucin, type 1701-W, supplied by the Wilson Laboratories, Chicago.

a loopful onto the surface of another plate. The cultures were all placed in a metal box containing a lighted candle and incubated at 37°.

When direct microscopic examination was desired, smears were made of the aqueous, ciliary body and lens immediately after the cultures.

The cultures were inspected carefully after 18 to 24 hours incubation and again after a second day's incubation. Colonies of gonococci were identified by smears stained by Gram's method and sugar fermentation reactions.

Results. Appearance of the infected eyes: Eyes inoculated late in the afternoon showed considerable inflammatory reaction by the following morning: injection of the bulbar conjunctiva, especially around the limbus, clouding of the cornea, and exudate in the anterior chamber. As time went on the conjunctival and ciliary injection grew more pronounced and vascularization of the cornea began. In some cases intra-ocular tension increased and even caused rupture of the bulb if it was not relieved by aspiration of fluid from the anterior chamber.

The results of cultures from different parts of each eye were not uniformly consistent, that is, the culture of one part of an eye might yield a confluent growth of gonococci although one from another part of the same eye contained few colonies or none. In general, cultures of the anterior surface of the lens most frequently contained the largest numbers of gonococci. Those of the ciliary body came next, and those of the aqueous and vitreous humors third and fourth in order of decreasing incidence. If a culture from any part of an eye contained any colonies of gonococci that eye was regarded as infected at the time of its enucleation.

TABLE I.
Infections Resulting from Inoculation of Different Numbers of Gonococci.

Approximate No. of gonococci	No. of eyes inoculated	No. of infections resulting	%
20,000,000	266	248	93
2,000,000	32	27	84
200,000	28	23	82
20,000	32	21	65
2,000	26	12	46
200	22	10	45
20	10	0	0

Size of inocula required to infect: To determine the proportion of infections resulting from inocula of varying sizes and also the minimal infective dose, eyes were injected with dilutions of the standard suspension of gonococci and cultured at the end of 24 hours.

As will be seen in Table I positive cultures were obtained at the end of 24 hours in 93% of the eyes injected with approximately 20,000,000 gonococci. The reason for the preponderant number of eyes receiving this particular inoculum was that they served as controls for a series of tests which will be reported in a subsequent communication. The percentage of infections diminished with the number of gonococci injected, but inoculation of 22 eyes with only 200 gonococci resulted in 10 infections.

Definite evidence that multiplication of the inoculated gonococci took place within the anterior chamber and its adjacent tissues was obtained from the number of colonies developing from measured portions of the aqueous and of suspensions of the macerated tissues of the eye.

Duration of the infection: To determine the length of time viable gonococci persisted in inoculated eyes the following experiment was carried out: The eyes of a series of rabbits were infected with approximately 20,000,000 gonococci each. At regular intervals, thereafter, animals were sacrificed and their eyes cultured.

Results. (See Table II) During the first week there was a progressive fall in the percentage of eyes from which viable gonococci could be cultivated. At the end of one week over a third of the eyes were still positive. Subsequent observations which were made at weekly intervals were too few to treat statistically, but the proportion of positive infections continued at about the same rate. These results indicate that this infection becomes chronic in approximately a third of the eyes and may persist for as long as 14 weeks—the maximum period of observation.

It should be noted that the data contained in Table II represent the results of cultures made at autopsy. Some of the negative cultures came from eyes which had been aspirated

TABLE II.
Duration of Infection
As determined by recovery of viable gonococci from eyes at various times after inoculation.

Time after inoculation	No. of eyes cultured	No. of eyes positive	% positive
1 day	10	10	100
2 days	11	10	91
3 "	12	9	75
4 "	12	7	58
5 "	15	7	46
6 "	24	11	45
7 "	23	8	35
2 wks	6	2	
3 "	4	2	
4 "	4	1	
5 "	4	1	
6 "	4	2	
7 "	2	1	
8 "	6	3	
9 "	4	2	
10 "	10	3	
11 "	6	2	
12 "	2	0	
14 "	2	2	

at various times during life and found to contain living gonococci.

Discussion. The lesion produced in the anterior chamber of the rabbit's eye by the introduction of gonococci is regarded as a genuine infection for the following reasons: Viable gonococci were recovered from 93% of the inoculated eyes at the end of 24 hours and from 39% at the end of 7 days. The inoculated gonococci were found to invade the neighboring tissues (lens and ciliary body) and definite evidence of multiplication was obtained. The infection could be transmitted in series by transfer of a drop or two of aqueous humor, but this method of animal passage was impracticable for routine experimental purposes, because contaminants could not be detected until after the transfer had been made. In approximately ⅓ of the inoculated animals the infection became chronic and persisted until the end of the longest period of observation which was 14 weeks.

This infection has been found to lend itself to the study of local and systemic therapy of gonococcal infection and to the testing of gonococcidal agents *in vivo*.

Summary. A method is described for the production of a localized gonococcal infection in the rabbit's eye by inoculating gonococci into the anterior chamber. Gonococci were found to invade the tissues of the eye, to multiply there and to produce severe inflammatory reaction. In approximately ⅓ of the animals the infection became chronic and viable gonococci persisted as long as 14 weeks, the maximum period of observation.

The authors are indebted to Dr. Arlington C. Krause and Dr. Maurice J. Drell of the Department of Surgery for many helpful suggestions.

14876

Influence of Muscle Pain on Electrical Resistance of the Human Skin.*

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It was shown in a series of investigations from this laboratory[1-4] that muscle pain greatly influences the somatic nervous system at spinal and supra-spinal levels. Autonomic changes occur also under these conditions as evidenced by pupillary dilatation which is based on an inhibition of the tone of the third

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

1 Gellhorn, E., and Thompson, L., *Am. J. Physiol.*, 1944, **142**, 231.

2 Gellhorn, E., and Thompson, L., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 209.

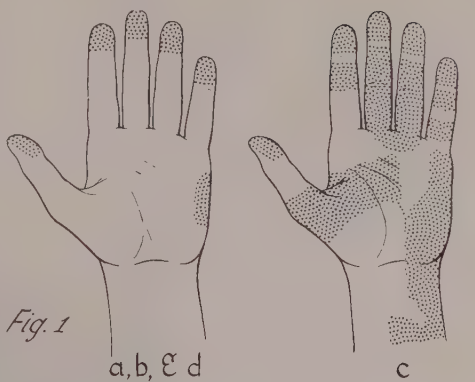
3 Gellhorn, E., *Journal Lancet*, 1944, **64**, 242.

4 Thompson, L., and Gellhorn, E., PROC. SOC. EXP. BIOL. AND MED., 1945, in press.

nerve nucleus.[5] Although the sympathetic innervation of the pupil remained unchanged it seemed likely that other structures might reveal signs of altered sympathetic innervation under the influence of muscle pain. The experiments reported in this paper give positive evidence for this assumption.

Method. Sixteen normal adults served as subjects in this series. The skin resistance of the left hand and in some experiments of face and foot was determined by the method of Richter[6,7] and the area of low resistance was mapped out. Thereafter the right forearm and hand or the right lower leg were made ischemic by means of a blood pressure cuff applied above the elbow or knee and inflated to a pressure of 200 mm Hg. In other experiments a tourniquet was applied to the shoulder. After the effect of ischemia on the low resistance area had been determined ischemic pain was induced by repeated movements of the ischemic muscles (Lewis[8]) or, in a few instances, by immersion of the ischemic hand and forearm in water of 115°F (Harpuder and Stein[9]). As soon as pain became manifest the area of low skin resistance was again determined. As was pointed out in previous papers the pain disappeared almost immediately upon restoration of circulation. A second control experiment was performed about 10 minutes after cuff or tourniquet had been removed.

Results. The results are best described by reproducing the actual records of a typical case. Fig. 1 shows that the low resistance area is restricted on the volar surface of the hand to the tips of the fingers and thumb, and to a small part of the hypothenar eminence. Ischemia does not alter the boundaries of the low resistance area, but under the influence of ischemic pain originating in the right biceps the low resistance area covers the greater part



Effect of ischemic pain in the right biceps on the skin resistance of the left hand: (a) control; (b) ischemia of the right arm, shows this same area of low resistance; (c) ischemic pain of right biceps, shows extension of low resistance area; (d) control, 10 minutes after restoration of circulation, shows the same low resistance area as in a.

of the volar surface of hand and fingers. A control experiment performed about 10 minutes later shows that the effect is completely reversible.

Such experiments were carried out on several persons 2 to 4 times with very similar results. The area of low resistance was very constant in spite of the fact that, in some instances, several weeks had passed between the individual experiments. The effect of pain showed only slight quantitative variations in the extent of the low resistance area.

Fig. 2 shows the effect of ischemic pain induced in the right foot on the skin resistance of the left hand on the same subject. Under these conditions the area of low resistance is extended to the greater part of the volar surface of hand and fingers and to the flexor surface of the forearm up to a line about 10 cm above the wrist.

Experiments on other subjects confirm these results, showing that the volar surface of the fingers, palm, and forearm are the areas which are involved in decreasing degree in conditions of muscle pain.

These effects are likewise observed if the ischemic fingers or hand and forearm are immersed in water of 115°F. Harpuder and Stein state that pain is largely of cutaneous origin if the fingers alone are immersed but that cutaneous and muscle pain are evoked

[5] Ury, B., and Gellhorn, E., *J. Neurophysiol.*, 1939, **2**, 268.

[6] Richter, C. P., and Woodruff, B. G., *Bull. Johns Hopkins Hosp.*, 1942, **70**, 442.

[7] Richter, C. P., Woodruff, B. G., and Eaton, B. C., *J. Neurophysiol.*, 1943, **6**, 417.

[8] Lewis, T., *Pain*, N.Y., 1942.

[9] Harpuder, K., and Stein, I. D., *Am. Heart J.*, 1943, **25**, 429.

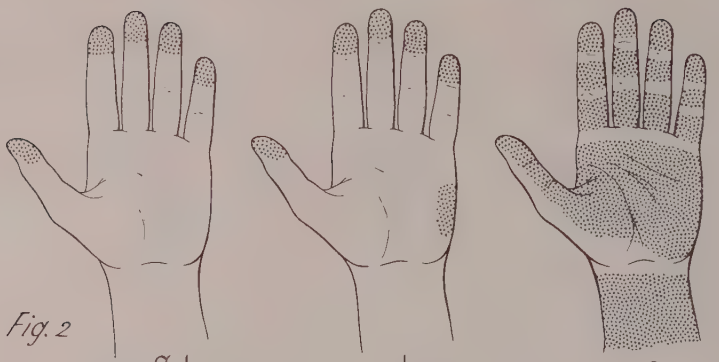


Fig. 2

The effect of ischemic pain in the right foot on the skin resistance of the left hand: (a) control; (b) ischemia, shows the same distribution of low resistance area except for a small area appearing on the hypothenar; (c) ischemic pain of right foot, shows increase in low resistance area of left hand; (d) control, 10 minutes after restoration of circulation, shows that low resistance area is the same as in (a).

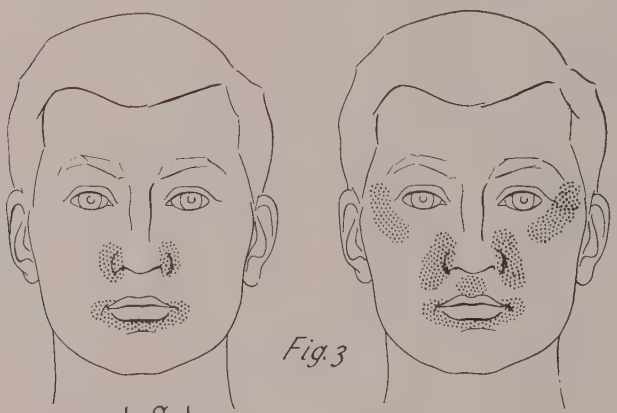


Fig. 3

Effect of ischemic pain (arm) on the skin resistance of the face: (a) control; (b) ischemia of right arm, the low resistance area remains unchanged; (c) ischemic pain of right arm, low resistance area of face increases slightly: (d) control, 10 minutes after restoration of circulation, low resistance area as in (a) and (b).

if hand and forearm are immersed at the same time. Other skin areas may likewise be involved, but to a much smaller degree than the volar surface of the hand. Thus, in some instances it was found that an area of low resistance appeared on the dorsum of the hand, and in another subject, the low resistance area extended on the flexor side up to the shoulder. On the dorsal and plantar surface of the foot appeared after pain small areas of low resistance which were absent under control conditions and during the period of ischemia. Changes in the resistance of the skin of the face were slight in spite of the fact that vasomotor effects (flushing) were quite common as a result of pain. The effect consisted in a slight enlargement of the area of low resistance observed under control conditions and in the appearance of additional areas formerly showing relatively high resistance (Fig. 3).

Discussion. According to the investigations

of Richter and collaborators the area of low resistance in the skin is related to sympathetic innervation and to the distribution of sweat glands. These areas expand in conditions associated with increased sympathetic activity such as muscular exercise and emotional excitement, and decrease in sleep. Cold and warm tub baths respectively decrease and increase the size of the low resistance area. In the light of these observations the reversible enlargement of the areas of low resistance under the influence of muscle pain indicates that the latter increases sympathetic discharges. This effect is likewise observable under conditions of cutaneous pain. There is, however, another interpretation possible which could point to the relationship between these effects of pain and emotion and attribute to the emotional disturbances induced by pain the changes described in this paper. This objection is held invalid for two reasons: first, the persons on whose experimental records this investigation is based were selected from a larger group because of their emotional stability and low sympathetic excitability as indicated by the size of the low resistance area of the skin. Richter mentions that the low resistance area comprises the whole volar surface of the skin. We find this to be true only for rather excitable persons who were excluded from this series for this reason. Secondly, the experiments were performed repeatedly on the same subjects with very similar results although emotional disturbances would be apt to decline on repetition.

Richter points out that the distribution of the low resistance area in the skin does not reflect either the distribution of peripheral nerves or that of spinal dermatomes, but rather that of cortical patterns of autonomic innervation. This statement seems to be valid for the changes seen as a result of muscle or cutaneous pain. It is of interest to mention in this connection an earlier investigation which demonstrated an increased responsiveness of the motor cortex to electrical stimulation as a result of muscle pain.[1]

Summary. Muscle or cutaneous pain induced by movements of muscles in ischemia or immersion of ischemic fingers in hot water increases the area of low resistance of hand, forearm, and face reversibly. The effects indicate increased sympathetic discharges. The pattern suggests the involvement of supraspinal mechanisms.

14877

Increased Germicidal Efficiency of Iodine in the Presence of an Oxidation-Reduction System.

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In a previous communication[1] it was shown that inorganic salts of iron, manganese, and tin, when tested individually, exhibited very little or no germicidal activity against the test organism *Staphylococcus aureus*. However, if an oxidized and a reduced salt, such as ferric and ferrous sulfates, were dissolved in water to form an oxidation-reduction system, the solution exhibited a pronounced germicidal action. The phenomenon was shown to be a function of the positive metallic ions; the negative ions apparently played no part in the reaction. For example, a mixture of sodium sulfate and sodium sulfite exhibited no increase in germicidal activity over that of the same salts tested individually.

The salts must be combined in certain definite proportions for maximum germicidal activity to occur. In the case of the chlorides of iron, the most effective germicidal activity occurred when the salts were mixed in the proportion of two moles of ferric chloride and one

[1] Guest, Howard L., and Salle, A. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 272.

mole of ferrous chloride. The sulfates of iron behaved in a similar manner, the most effective combination being in the proportion of one mole of each salt. Stannous and stannic chlorides also functioned most effectively in the proportion of one mole of each.

The germicidal effect was not limited to combinations having a common metallic ion. Metallic salts of different cations and anions were crossed with the same general effect. The important consideration was to have an oxidation-reduction system for increased germicidal activity to occur.

In a later communication[2] it was shown that the addition of an appropriate metallic salt to an organic germicide, to produce an oxidation-reduction system, resulted in a great increase in the efficiency of the germicide. For example, the addition of ferric sulfate to phenol increased the germicidal potency of the latter 18 times. The addition of another oxidation-reduction system, composed of a mixture of ferrous and ferric chlorides, to the ferric sulfate-phenol combination resulted in a further increase in the potency of the latter approximately 45 times.

Experimental. The oxidation-reduction (o-r) system used was composed of equimolar quantities of manganous sulfate ($MnSO_4 \cdot H_2O$) and ferric sulfate ($Fe_2(SO_4)_3 \cdot 6H_2O$). These two salts produced a stable o-r solution. The solution was observed for over a year without any change in color, appearance, and strength. The solution was prepared by dissolving 50 g of the combined salts (12.5 g $MnSO_4 \cdot H_2O$ + 37.5 g $Fe_2(SO_4)_3 \cdot 6H_2O$) in sufficient distilled water to make 1000 cc. This was considered a 1:20 dilution.

A stock solution of iodine was prepared by dissolving 5 g of iodine and 10 g potassium iodide in sufficient distilled water to make 1000 cc. This was considered a 1:200 dilution.

The 24-hour cultures of *Staphylococcus aureus* and *Eberthella typhosa* were standardized in a photoelectric colorimeter before use. Turbidity standards of barium sulfate were prepared according to the McFarland nephelometric method. The cultures were diluted with broth until they gave the same reading as a No. 7 standard.

TABLE I.
Toxicity of Iodine and the Oxidation-Reduction Solution for Embryonic Chick Heart Tissue Fragments.

Solution	Killing dilution for tissue
O-R solution alone	1:2000
Iodine + 1:3000 o-r	1:3750 iodine

All bactericidal and tissue toxicity tests were carried out in a shaking water bath at a temperature of 37°C and the exposure period was 10 minutes.

The effect of the o-r solution alone and with iodine on embryonic chick heart tissue fragments was determined by the tissue culture technic.[3] A dilution of 1:2000 o-r solution killed the tissue fragments. In order to evaluate the killing dilution of iodine in combination with the o-r system, it was necessary to use a more dilute solution of the metal salts. Accordingly, a 1:3000 dilution of the o-r solution was employed in the test. Iodine in combination with a 1:3000 dilution of the o-r solution killed chick heart tissue fragments in a dilution of 1:3750 of iodine. The results are given in Table I.

The killing dilutions of iodine for *Staph. aureus* and *E. typhosa,* in the absence of organic matter, were 1:20,000 and 1:17,500 respectively. In the presence of organic matter the dilutions were reduced to 1:2000 for *Staph. aureus* and 1:1750 for *E. typhosa.* The killing dilutions of the o-r solution for *Staph. aureus* and *E. typhosa* in the absence of organic matter were 1:75 and 1:1000 respectively. In the presence of organic matter these figures became 1:55 for *Staph. aureus* and 1:250 for *E. typhosa.* Since a 1:75 dilution of the o-r solution killed *Staph. aureus* it was necessary to combine a more dilute solution with iodine to make it possible to find the killing dilution of the combination. Iodine combined with a 1:100 o-r solution killed *Staph. aureus* in a dilution of 1:450,000 of iodine in the absence of organic matter, and 1:30,000 in its presence. Iodine combined with a 1:3000 o-r solution (the same strength as used in the tissue toxicity tests) killed

[2] Salle, A. J., and Guest, Howard L., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 26.

[3] Salle, A. J., McOmie, W. A., Shechmeister, I. L., and Foord, D. C., *J. Bact.*, 1939, **37**, 639.

TABLE II.
Killing Dilution of Iodine Alone and in Combination with an Oxidation-Reduction System.

Solution	Killing dilution: Without organic matter	Killing dilution: With organic matter*
Staphylococcus aureus		
Iodine alone	1:20,000	1:2,000
O-R solution alone	1:75	1:55
Iodine + 1:3,000 o-r	1:80,000 I	1:5,000 I
Iodine + 1:100 o-r	1:450,000 I	1:30,000 I
Eberthella typhosa		
Iodine alone	1:17,500	1:1,750
O-R solution alone	1:1,000	1:250
Iodine + 1:3,000 o-r	1:60,000 I	1:4,500 I

* The organic matter consisted of a mixture of 3 parts embryonic extract and 1 part defibrinated horse serum.

The embryonic extract was prepared by mincing 12-days-old chick embryos, diluting the pulp to 5 times its volume with Tyrode's solution, and centrifugating the mixture. The clear supernatant liquid is the extract.

Staph. aureus in a dilution of 1:80,000 and *E. typhosa* in a dilution of 1:60,000, both in the absence of organic matter. In the presence of organic matter *Staph. aureus* was killed in a dilution of 1:5000 and *E. typhosa* in a dilution of 1:4500 of iodine. The results are recorded in Table II.

Toxicity indexes may be calculated from the results of the bactericidal and tissue toxicity tests. The Toxicity Index may be defined as the ratio of the highest dilution of germicide required to kill embryonic chick heart tissue fragments in 10 minutes at 37°C to the highest dilution required to kill the test organism under the same conditions. Theoretically, an index less than one means that the germicide is more toxic to bacteria than to tissue; an index greater than one means that the germicide is more toxic to tissue than to bacteria. The smaller the Toxicity Index the more nearly perfect the germicide. The results are recorded in Table III.

It may be seen that although the toxicity indexes of the o-r solution are higher than those of iodine alone[4] the toxicity indexes of the combinations of iodine and o-r solution are considerably lower than those of the two components tested individually. The figures show that iodine with the o-r solution is 4 times more efficient than iodine alone.

This observation possesses considerable importance. It means that less iodine is required to produce an effective germicidal solution. A 1:500 solution of iodine with a proportionate amount of the o-r salts should eliminate entirely any tendency of iodine to burn or irritate and still be a powerful germicide. Because of its very low tissue toxicity, freedom from bacteriostasis and cumulative action,[5] such a solution should prove of great value for clinical application as well as for general use.

After this work was completed it was noted that the mixture of iodine and the o-r salts produced a slight precipitate on long standing. By using an o-r solution composed of the chlorides of manganese and iron instead of the sulfates precipitation did not occur.

The o-r solution now recommended is prepared by dissolving 50 g of combined salts (13.4 g $MnCl_2 \cdot 4H_2O$ + 36.6 g $FeCl_3 \cdot 6H_2O$) in sufficient distilled water to make 1000 cc. This was considered a 1:20 dilution.

Iodine in combination with a 1:3000 dilution of the new o-r solution (o-r II) killed *Staph. aureus* in a dilution of 1:120,000 and *E. typhosa* in a dilution of 1:50,000 of iodine. Iodine in combination with a 1:3000 dilution of the o-r II solution killed chick heart tissue fragments in a dilution of 1:5000 of iodine. The results are recorded in Table IV.

The figures show that the o-r II system in combination with iodine gave a smaller Toxicity Index against *Staph. aureus* and a larger one against *E. typhosa*. Since *Staph. aureus* is considered a more important organism for the evaluation of germicides it may be concluded that the new o-r solution not only produced a stable solution with iodine but also improved the efficiency of the latter.

Conclusions. In the absence of organic matter, iodine killed *Staphylococcus aureus* in a dilution of 1:20,000 and *Eberthella typhosa* in a dilution of 1:17,500 in 10 minutes at 37°C. In the presence of organic matter the dilutions were reduced to 1:2000 for *Staph. aureus* and 1:1750 for *E. typhosa*.

Iodine dissolved in a 1:3000 dilution of an oxidation-reduction solution (composed of a

[4] Salle, A. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 141.

[5] Salle, A. J., *J. Pharm. and Exp. Therap.*, 1943, **79**, 271.

TABLE III.
Toxicity Indexes of Iodine Alone and in Combination with an Oxidation-Reduction System.

Solution	Killing dilution: Tissue (A)	Killing dilution: *Staph. aureus* (B)	Killing dilution: *Eberthella typhosa* (C)	Toxicity index: *Staph. aureus* A/B	Toxicity index: *Eberthella typhosa* A/C
Iodine alone	1:4,000	1:20,000	1:17,500	0.20	0.23
O-R solution alone	1:2,000	1:75	1:1,000	26.60	2.00
Iodine + 1,3000 o-r	1:3,750 I	1:80,000 I	1:60,000 I	0.05	0.06

TABLE IV.
Toxicity Indexes of Iodine in Combination with an Oxidation-Reduction System (O-R II).

Solution	Killing dilution: Tissue (A)	Killing dilution: *Staph. aureus* (B)	Killing dilution: *Eberthella typhosa* (C)	Toxicity index: *Staph. aureus* A/B	Toxicity index: *Eberthella typhosa* A/C
Iodine + 1:3,000 o-r II	1:5,000	1:120,000	1:50,000	0.042	0.1

mixture of one mole each of manganous sulfate and ferric sulfate) killed *Staph. aureus* in a dilution of 1:80,000 and *E. typhosa* in a dilution of 1:60,000, both in the absence of organic matter. In the presence of organic matter the killing dilutions were 1:5000 and 1:4500 respectively.

A dilution of 1:2000 of the o-r solution killed embryonic chick heart tissue fragments in 10 minutes at 37°C. Iodine dissolved in a 1:3000 o-r solution killed the tissue fragments in a dilution of 1:3750 of iodine.

Toxicity indexes may be calculated by dividing the highest dilution of iodine required to kill the tissue by the highest dilution required to kill the test organism under the same conditions. The Toxicity Index of iodine + 1:3000 o-r solution was 0.05 for *Staph. aureus* and 0.06 for *E. typhosa*. The Toxicity Index of iodine alone was 0.2 for *Staph. aureus* and 0.23 for *E. typhosa*. Theoretically, an index less than one means that the germicide is more toxic to bacteria than to tissue; an index greater than one means that the germicide is more toxic to tissue than to bacteria. The smaller the index the more nearly perfect the germicide.

When iodine was dissolved in a 1:3000 o-r solution composed of manganous and ferric chlorides instead of the sulfates, the Toxicity Indexes were 0.042 for *Staph. aureus* and 0.1 for *E. typhosa*.

The importance of this observation is discussed.

14878

Attempts to Find Poliomyelitis Virus in Fish.*

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Each year since 1932 we have searched in vain for poliomyelitis virus in the gastrointestinal contents of fish caught in waters near epidemic areas with consistently negative results. There might have been several reasons for our failures. (1) The gastrointestinal contents used in our experiments

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could not always be rendered completely sterile and the pyogenic infection which usually followed intracerebral injections at that time may have overshadowed any effects from the virus that might have been present. (2) We might not have been fortunate enough to have obtained the fish from the place harboring the virus.

The first difficulty was solved when Kramer *et al.* showed that ether would kill organisms in the stool, but not appreciably affect the virus present. The 1941 epidemic of poliomyelitis in Cleveland began during the summer in the northeastern section of the city not far distant from Lake Erie. It also appeared about the same time in several isolated spots in the northeastern section of the state bordering the lake.

Fish were caught in waters infected by sewage drainage. Three such localities along Lake Erie were investigated in 1941, namely, polluted Brook D along the banks of which flies were collected and which carried the virus, and the mouths of two small rivers which emptied into Lake Erie.

The gastrointestinal tracts of several fish (mostly carp) were milked free of their contents, to which anesthetic ether was added to approximately 13% of the total volume. The ether was drawn off by vacuum. If the material was not sterile after standing, the procedure was repeated. The final sterile supernate was injected into 2 *M. mulatta* monkeys as follows: 40 cc intraperitoneally (I.P.) 5 times, 1 cc intranasally (I.N.) 13 times, 1 cc intracerebrally (I.C.) 2 times. One animal became furred. The other had weakness of one side, probably traumatic. The microscopic sections of its lumbar cord showed destruction of anterior horn cells, but no typical vascular reaction. Transfer attempts were negative. This and 2 subsequent experiments were negative.

Later the contents of the gastrointestinal tract obtained from at least 4 dozen fish, caught at the mouth of one of the rivers, were combined, centrifuged and the supernate treated with ether. The material was placed in a "Visking" sack and concentrated by fanning. On 10/3/41, monkey I was injected as follows: 40 cc I.P., 10 cc S.Q., 1.0 cc I.N., and 1.0 cc I.C. Seven days later, the animal had weakness in the legs; it was clumsy on the 13th day and on the 14th day it was sacrificed.

The second monkey was injected on 10/20/41 with a suspension made from the cord obtained from the first animal. The doses were: 1 cc I.C., 1.0 cc I.N, and 30 cc I.P. Two days later, a similar set of injections was given. The animal had a tremor and became slow and deliberate in its movements; on the 5th day, it had weakness in both legs. It was sacrificed on the 14th day. On 11/22/41, 1 cc of a 20% unpurified suspension of its lumbar cord was injected I.C. into another animal (monkey III). Five days later the muscles of the arms and left leg were week and on the 7th day the left leg was dangling. On the 7th and 15th days, it was given another injection I.C. with no added results. The animal was sacrificed on the 25th day. Sub-transfer of its cord into another animal (monkey IV) was negative.

On 11/26/41, a large batch of fish was caught in harbor F. The guts were prepared in the usual way and injected into a monkey (V) on 12/1/41. It received 30 cc I.P., 30 cc S.Q., and 2 cc I.N. On the 11th day, the animal had weakness of the right leg, but it did not seem typical and it recovered completely. It was reinjected with the same dosage of material on 12/5/41, observed for 44 days and sacrificed. The microscopic sections of the cord were negative.

Although paresis was found in monkeys I and II and paralysis in monkey IV, there was no evidence that these conditions were due to poliomyelitis virus.

Subsequent attempts to obtain virus from fish caught in (a) Lake Superior, (b) Lake Michigan, and (c) on the Canadian side of Lake Erie were failures.

Our next experiments were directed toward determining whether fish possess natural neutralizing antibodies against poliomyelitis virus. The scavenger carp was selected for this work. Blood was obtained and neutralization tests made in all instances by adding the questionable neutralizing antiserum, as was, to 10% poliomyelitis virus of known potency. The virus used in all experiments was Flexner M.V. strain. *M. mulatta* monkeys were used

as the test animal. Cotton rats and Flexner M.V. strain adapted to this species were used in the final experiment only. In 6 experiments, no neutralizing antibodies were demonstrated in the blood specimens of 6 carp, indicating possibly that carp do not develop antibodies because either they are never exposed or are naturally resistant to poliomyelitis virus.

We next tried to infect carp artificially. There were difficulties in obtaining live fish, or at least partly frozen fish that would survive. The iced fish that arrived all winter could not be easily revived. It was not until the spring of 1942 that we obtained carp for our purpose. There was some difficulty in keeping them alive and every one of several batches died, even though our tank was large and kept filled with water running constantly. Our city water is heavily chlorinated in the springtime. We were advised to paraffin the sides of the copper tank, to fill it with water and to allow it to remain there 24 hours, and then to start the current going slowly. Eight fish, weighing about 3 pounds each, were placed in the tank prepared in this manner. They all lived. Two of the fish were sacrificed. Their stools were tested for virus and their bloods for neutralizing antibodies. The results were negative.

The 6 remaining fish were fed virus in various ways—in balls of bread, whole pieces of cord, etc. Finally, the virus was merely ground up and poured into the tank, after which the water became milky white in color. The water drain was plugged for a few days and the running water stopped so that the virus material would not be dispersed. Two months later 2 fish were killed. Tests were made for neutralizing antibodies and a search was made for virus in the gastrointestinal tracts. Again, the results were negative.

The fish left were tested 2 months later and again neutralizing antibodies were not found in the blood serum. Three months passed and one fish was sacrificed. There was neither evidence of virus in the gastrointestinal tract nor evidence of neutralization antibodies in the blood. One fish was accidentally killed.

The 2 remaining carp were given 1 cc of 10% virus suspension intraabdominally every 10 days for 10 doses, starting on 11/25/42. The fish were bled on 5/26/43 and on 11/27/43. Neutralization tests of the blood serums were made in monkeys with negative results. Since antibodies may appear over one year later in some species under certain circumstances,[1] the fish were again bled on 6/11/44, about a year and a half after immunization had been started. Once more the results were negative. Cotton rats were used in this experiment (10 controls injected with a mixture of virus and saline, and 10 with a mixture of virus and immune serum). All of the animals had paralysis and died, these experiments also being negative. It is most unusual that the virus antigen did not produce serum antibodies in the fish when it does so easily in other species. It raises most interesting speculations as to why they are immune.

Thus, we were not able to demonstrate the presence of virus in the carp. Nor could we with the methods and the amounts used immunize these fish to the point where they would show neutralizing antibodies; neither could we demonstrate live virus in their stool contents.

[1] Toomey, John A., *Am. J. Dis. Child.*, 1937, **53**, 1492; 1938, **55**, 1261.

14879

Direct Isolation of Influenza Virus in Chick Embryos.

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Primary isolation of influenza virus in chick embryos has become a useful technique in the past several years largely through the work of Burnet on the advantages of inoculation into the amniotic sac,[1] and through the use of hemagglutinins as a means of detecting the virus.[2,3] Recently, Rickard, Thigpen, and Crowley[4] have shown that influenza virus may be isolated by inoculation of untreated throat washings into the allantoic sac. With amniotic inoculation it has been necessary to filter the throat washing to prevent embryonic deaths from bacterial contamination, but in the work to be reported this step has been eliminated through the use of penicillin, and it will be shown that this change makes the chick embryo isolation procedure as sensitive as any now known.

Methods. Throat washings were obtained from influenza A patients in the 1943-44 epidemic, mainly in Army hospitals. The patients gargled with 15 cc of plain broth, which was then kept at a temperature of —72° C until tested. Five methods were used for the isolation or detection of influenza virus in the washings: (1) Penicillin was added to throat washings to a final concentration of 125 units per cc. This mixture was inoculated into the amniotic sacs of 13-day-old chick embryos, 0.2 cc per egg. After incubation at 35° C for 4 days the amniotic fluids were removed and tested for guinea pig and chicken cell hemagglutinins. (2) Throat washings were filtered through 700 mμ collodion membranes and the filtrates were inoculated into the amniotic sac of 13-day-old embryos, which were further treated as in (1). (3) Untreated washings were inoculated into the allantoic sac of 11-day-old chick embryos, and after 2 days' incubation at 37° C the allantoic fluids were removed, tested for hemagglutinins, and passed in series by the allantoic route for 5 passages.[4] (4) Penicillin, in a final concentration of 125 units per cc, was added to throat washings, which were further handled as in (3). (5) Ferrets were tested at 2 weeks for their antibody response to an intranasal inoculation of 1 cc of unfiltered throat washing.

For the amniotic inoculation both the method of Burnet[1] and a method described by Hirst[3] were used with equal success in respect to virus isolations. The former requires the making of a window while the latter does not. Whenever fluids were found that contained hemagglutinins, the material was passed in 10^{-3} dilution by the allantoic route, and 4 agglutinating units of virus (in allantoic fluid) were tested for inhibition by ferret immune sera (Lee and PR8). By this method all the strains isolated were shown to be influenza A. Ferret sera were tested by agglutination inhibition against the PR8 and Lee strains, and all the positive results indicated an infection with a strain of influenza A.

Results. During the past several years a search has been made for a chemical agent which would be bacteriostatic in eggs but which would not affect the viability of influenza virus. Such an agent was found in penicillin, 25 units of which was sufficient to inhibit the growth of bacteria introduced with throat washings into eggs. Parallel *in ovo*[5] titrations of stock strains of influenza virus in plain broth and broth containing 125 units of penicillin per cc showed no effect of the penicillin on the viability of the virus.

The published work on chick embryo iso-

[1] Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18**, 353.

[2] Burnet, F. M., Beveridge, W. I. B., Bull, D. B., and Clark, E., *Med. J. Australia*, 1942, **2**, 371.

[3] Hirst, G. K., *J. Immunol.*, 1942, **45**, 293.

[4] Rickard, E. R., Thigpen, M. P., and Crowley, J. H., *J. Immunol.*, 1944, **49**, 263.

[5] Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

TABLE I.
Comparison of Different Methods of Detecting or Isolating Influenza Virus from Throat Washings.

		Method of detection				
	No. of throat washings	Throat washing plus penicillin into amniotic sac	Ferret immune response	Throat washing filtrate into amniotic sac	Throat washing plus penicillin into allantoic sac	Untreated throat washing into allantoic sac
	4	+	+	+	+	+
	4	+	+	+	+	—
	9	+	+	+	—	—
	13	+	+	—	—	—
	3	+	—	—	—	—
	12	—	—	—	—	—
	—	—	—	—	—	—
Total positive	33	33	30	17	8	4

lation of influenza virus shows marked discrepancies in the success of various methods and does not permit a valid comparison,[2-7] because throat washings vary so much in different epidemics. For this reason the 45 throat washings available were each tested for influenza virus by 5 different methods. The results of these tests are given in Table I.

There were 12 completely negative washings as tested by all methods. The inoculation of penicillin-treated washings into the amniotic sac was clearly the most sensitive method of virus detection, and no washings positive by any other method failed to yield virus by this technique on the first egg passage. Three washings yielded virus even though negative antibody responses were obtained in ferrets, a procedure which previously provided the most delicate test. The percentage of positive eggs with different washings varied from 15 to 100, with an average of 65. No throat washings were encountered in which penicillin failed to control bacterial growth. In confirmation of Burnet and Bull[8] it was found that guinea pig hemagglutinins usually (but not always) were present in higher titer than chicken cell agglutinins. In our experience 13-day embryos were vastly superior to 11-day embryos for obtaining positive results. Filtration of throat washings, as has been previously shown[3], removed detectable virus from about half the samples.

The allantoic route of inoculation was markedly less sensitive than any other method, yielding only 4 positive results with untreated washings; but it gave somewhat better results when penicillin was added to the inoculum. With the latter group all the positive results were obtained on the first passage. It is possible that the bacterial contamination that always occurs on the inoculation of untreated washings may have interfered (in 4 instances) with the propagation of the virus, since virus was detected in these washings by allantoic inoculation in the presence of penicillin. Allantoic takes occurred only with throat washings which also gave positive filtrates by the amniotic route, and it is fair to assume that the success of the allantoic method depends on the presence of a relatively high concentration of virus in the inoculum.

Acute and convalescent sera of the individuals from whom washings were obtained were tested in 35 instances. In 25 cases both serological and throat-washing results were positive, and in 7 cases both were negative. Two patients gave a positive serological response and negative throat washings, while 1 individual had a negative serological response but the washings did yield virus. There was agreement in the results of the 2 tests in 32 of 35 instances.

In summary, the allantoic route of inoculation was found to be advantageous for the chick embryo isolation of influenza virus chiefly because of its technical simplicity; however, the method fails to detect virus in many

[6] Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Australian J. Exp. Biol. and Med. Sc.*, 1944, **22**, 1.

[7] Andrewes, C. H., and Glover, R. E., *Lancet*, 1944, **2**, 104.

[8] Burnet, F. M., and Bull, D. B., *Australian J. Exp. Biol. and Med. Sc.*, 1943, **21**, 231.

known positive washings. While technically more difficult, the inoculation of penicillin and throat washing into the amniotic sac proved to be a highly satisfactory method of detecting influenza A virus and was the most sensitive of present-day methods in this experiment.

14880

Influence of Protein and Thiamine Intake on Pathologic and Weight Changes Produced by Atabrine.*

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The oral administration of atabrine produces necrosis and replacement fibrosis of the liver and myocardium in the rat.[1-3] A recent report[4] indicates that the toxic effects of atabrine upon the liver, as measured by an increase in plasma fibrinogen, can be largely prevented by a high protein-low fat diet. The present investigation was undertaken to determine the effect of the protein and thiamine content of the diet upon the hepatic and myocardial lesions in the rat which result from atabrine administration. The concomitant effect upon growth was also studied.

Experimental. Effect of Protein Intake. Young male rats of the Carworth strain were segregated into 8 groups of like average weight (123 g) and were maintained on high or low protein diets. In addition several of the groups received atabrine as indicated in Fig. 1. Four rats were employed in each of the control groups and 20 rats in each of the groups receiving atabrine.

The two experimental diets used had the following composition:

Low Protein—vitamin free casein, 6; dextrose, 78; hydrogenated vegetable fat, 8; cod liver oil, 2; mazola, 1; dried beef liver, 1; salt mixture USP XI No. 1, 4; supplemented with 0.8 mg each of thiamine, riboflavin, and pyridoxine; 8.0 mg each of nicotinamide and calcium pantothenate and 100 mg of choline chloride per 100 g of diet.

High Protein—This diet was identical with the low protein diet except that 24% of the dextrose was replaced by 24% casein, thus raising the casein content to 30% and lowering that of the dextrose to 54%.

The rats were allowed to equilibrate on these diets for a period of one week prior to dosing with atabrine. All animals receiving the drug were dosed daily by stomach tube with 45 mg per kg (5% of the L.D. 50). The experiment was terminated after 48 days (41 days of atabrine administration), at which time the rats on the low protein diet were in a debilitated condition.

At autopsy the degree of hepatic damage was estimated grossly by the amount of the entire liver which was necrotic. The heart, after fixation in 4% formaldehyde solution, was cut in half by frontal section. Histologic sections were made parallel to the cut surfaces of both halves and stained with hematoxylin-eosin. The degree of myocardial damage was graded as slight or moderate. Rats with "slight" involvement had only an occasional focus of necrosis; in those with "moderate" involvement several areas of the myocardium were affected.

Atabrine administration had a marked depressing effect upon weight as evidenced by the difference in the growth of rats in Groups

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the Merck Institute for Therapeutic Research.

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TABLE I.
Degree and Extent of Liver and Myocardial Damage of Atabrine Dosed (45 mg per kg) Male Rats on High and Low Protein Diets.*

Group	No. of rats	Diet	Liver damage: % with necrosis	Liver damage: Avg degree of involvement	Myocardial damage: % with necrosis and fibrosis	Myocardial damage: Avg degree of involvement
2	21	Low protein *ad lib.*	95	severe	48	slight
5	18	High protein *ad lib.*	44	slight	83	slight to moderate
8	19	High protein restricted to group 2	84	slight to moderate	79	moderate

* The livers and hearts were normal in all *ad libitum* as well as restricted diet controls.

1 and 2 and in Groups 4 and 5 (Fig. 1). Furthermore, the animals in each of the atabrine-dosed groups weighed less than did their isocaloric controls (Groups 2 and 3; 5 and 6). Rats on the high protein diet *ad libitum* which were dosed with atabrine showed a greater depression in weight relative to their undosed isocaloric controls (43 g) than did the corresponding rats on the low protein diet (23 g).

The incidence and degree of myocardial necrosis and replacement fibrosis after atabrine administration were greater in the animals fed the high protein diet than in those fed the low protein diet (Table I). On the other hand, the highest incidence and degree of liver necrosis were observed in the animals receiving the low protein diet (Group 2, Table I.). Although a high percentage of the rats on the high protein diet, restricted in caloric intake to that of the low protein animals (Group 8), showed liver necrosis, the degree of involvement was less than that in the latter group. The rats receiving the high protein diet *ad libitum* (Group 5) showed the lowest incidence and degree of hepatic damage. The livers and hearts of all controls (not receiving atabrine) were normal.

Effect of Thiamine Intake. In another experiment the effects of a thiamine-low diet upon the toxicity of atabrine were studied. Adult rats maintained on 2 to 4 micrograms thiamine daily, were as resistant as were their controls to the pathological changes induced by atabrine administration (45 mg per kg).

Discussion. It has been shown by Goldschmidt, Vars and Ravdin[5] that a low protein diet will render the liver more susceptible to hepatoxic substances. More recently, Scudi and Hamlin[4] have demonstrated that a low protein diet aggravates the degree of atabrine toxicity in rats and dogs as measured by an augmentation in plasma fibrinogen, the values of which parallel closely the degree of

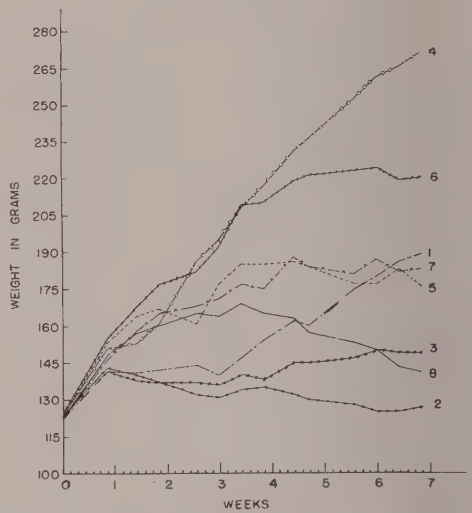

FIG. 1

Weight curves of groups of rats on high and low protein diets with and without atabrine.
Group 1—Low protein diet ad lib.
Group 2—Low protein diet ad lib. plus atabrine (45 mg/kg daily)
Group 3—Low protein diet; caloric intake restricted as in Group 2
Group 4—High protein diet.
Group 5—High protein diet ad lib. plus atabrine (45 mg/kg daily)
Group 6—High protein diet; caloric intake restricted as in Group 5
Group 7—High protein diet; caloric intake restricted as in Group 2
Group 8—High protein diet; caloric intake restricted as in Group 2 plus atabrine (45 mg/kg daily)

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hepatic damage in the rat.[6] In the studies herein reported a high level of dietary protein appeared to have a protective effect against the hepatic necrosis produced by atabrine (45 mg per kg). Hegsted, McKibbin and Stare[7] failed to observe hepatic necrosis or myocardial damage in rats receiving 40 mg % atabrine in the diet for a period of 6 months. The intake of the drug, however, was probably less than 40 mg per kg body weight due to the depressing effect of the compound upon appetite.

A low protein diet failed to aggravate the toxic effect of atabrine upon the muscle of the heart. On the contrary, the incidence and degree of myocardial damage were higher in the animals receiving a high protein diet, a finding in agreement with that of Nelson and Fitzhugh.[8]

The pathological changes produced by atabrine in the heart and in the liver were the same in rats maintained on a low thiamine diet as in those on a natural food ration. In this connection it is of interest to note that Follis[9] found that a thiamine deficiency was protective against the myocardial lesions produced by a deficiency in potassium. Hegsted *et al.*[10] reported that atabrine has a "thiamine sparing" action when fed to rats on a thiamine deficient ration.

The influence of other dietary constituents upon atabrine toxicity has recently been reported. Wright and Lillie[1] found that the tolerance to atabrine was not influenced by the addition of riboflavin to a stock ration. Hegsted, *et al.*[7] reported that the slow growth obtained on sub-optimal levels of riboflavin was further decreased by the addition of 40 mg % atabrine to the diet. On the other hand, these authors found that with diets suboptimal in vitamin A, the feeding of atabrine did not cause a further reduction in growth rate. Hegsted *et al.*[11] have also reported that the inclusion of atabrine in low choline diets at a level of 65 mg per 100 g of ration almost completely prevents hemorrhagic kidneys in the rat.

Summary. The hepatic damage resulting from continued atabrine administration was more severe in rats maintained on a low protein diet than in those on a high protein diet. On the other hand, the incidence and degree of myocardial damage were slightly greater in the rats fed the high protein diet. Thiamine deficiency had no effect upon the hepatic and myocardial damage produced by atabrine. The daily administration of atabrine to rats maintained on high and low protein rations resulted in a retardation of growth beyond that observed in their isocaloric controls which did not receive atabrine.

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14881

Influence of Electronarcosis on Secretory Activity of the Pituitary Gland.*

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Since 1938, electroshock therapy has been widely used in certain mental disorders, particularly in the involutional psychoses. More recently a related procedure, electronarcosis, has been studied in animals and in man with a view toward determining its suitability and therapeutic value in the treatment of schizophrenia.[1, 2] During the past several years investigations have been in progress in which various physiological effects of electronarcosis have been examined.[2–4]

Because of the position of the pituitary gland with respect to the path of the current and the possibility of either direct or indirect hypophyseal stimulation in consequence of the current application, it has seemed important to determine whether the pituitary secretory activity is influenced by electronarcosis. Guinea pigs and dogs have been used in the present investigation.

Methods. Electronarcosis was induced with 60 cycle sinusoidal current through electrodes placed over the temporal region according to the method previously described;[1] an initial current of high intensity (250 milliamperes for dogs, 50 milliamperes for guinea pigs) was applied for 30 seconds, after which the current was reduced to a level which just permitted a return of respiration (50 milliamperes for dogs, 7 milliamperes for guinea pigs). In the experiments on dogs the electrodes were padded brass discs 2 cm in diameter, moistened with salt solution and held in position by bandaging. To assure contact, an electrode jelly was used. In the guinea pig experiments smaller electrodes (1 cm) were used.

On completion of the series of electronarcoses in guinea pigs the animals were sacrificed, and their thyroids, adrenals, and ovaries were removed, weighed and compared with those of animals kept and killed under the same conditions but without electronarcosis.

Blood was taken from dogs before and after a series of electronarcoses, and the concentration of thyrotropic hormone was determined by the method of Smelser[5] using day-old Austra-white cockerels. The chicks were kept in an incubator in which a temperature of 33° C was maintained. Groups of about 10 chicks were used; a group of uninjected controls was kept in the same cage with each injected group.

Results. Symptoms of Electronarcosis. Dogs: The symptoms which occur when electronarcosis is applied to dogs have been comprehensively and adequately presented.[1, 2] *Guinea pigs:* Since electronarcosis in guinea pigs has not previously been described, the symptoms which they most uniformly show may briefly be considered. Immediately upon application of the high initial current, the animal falls over on one side, and almost at once develops a strong extensor spasm which usually is diminished to some extent after about 25 sec. Respiration is arrested during the entire 30 sec. application of high current, and does not return until about 15 sec. after the current has been reduced to the maintenance level. The high initial current pro-

* This study was supported by a grant from the Hixon Fund.

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duces regularly ejaculation in male guinea pigs, which does not occur in either dogs or man. Upon reducing the current to about 7 milliamperes most of the remaining extensor tone disappears, with the accompaniment, as a rule, of a few clonic twitches of the legs. Thereafter the animal becomes quiet, shows usually no response to severe pinching of the front feet, although in the hind feet the flexor reflex is frequently maintained at a low intensity. Pricking the skin over the body and head results in no response. Neither righting nor supporting reflexes are present. Considerable tone is found in nearly all guinea pigs, and frequently a more or less marked opisthotonic position is observed.

More like electronarcosis in man than in dogs, this quiet picture is gradually replaced in the course of the narcosis by an increasingly noticeable unrest or hyperkinesis on the part of the guinea pig. This can be suppressed to a greater or lesser extent by raising the current very slowly. However, in the guinea pig, secondary raises in the narcotizing current are often accompanied by a new convulsion, arrest of respiration, and frequently by death of the animal.

Recovery following discontinuation of the current passage is usually immediate. The animal generally shakes its head a few times and then hops away in an apparently normal manner.‡ In a number of experiments recovery was somewhat delayed, the animal having only weak righting reflexes and little or no supporting tone for several minutes, after which recovery became complete.

Effect of Electronarcosis on the Thyroid Gland. Four guinea pigs were each given a series of 10 daily electronarcoses of 2 min. duration. At the end of the series the animals were killed and their thyroid glands removed, weighed and prepared for histological study.§ The thyroids of the electronarcotized animals were found to be about 15% heavier than those of the controls. Histological examination showed a picture of moderate hypertrophy as evidenced by an increase in the height of the acinar epithelium from a mean of 5.5μ for the control animals to one of 8.1μ for those subjected to the series of electronarcoses. In a series of 10 min. electronarcoses on 2 animals it was found that the epithelial thickness was somewhat greater than that found in the series of 2 min. duration (Table I.) A marked withdrawal of the margin of the colloid and a conspicuous vacuolization of the colloid were also observed. The ovaries and adrenals of the electronarcotized guinea pigs were also from 25 to 30% heavier than those of the control animals.

TABLE I.
Influence of Electronarcosis on the Thyroid Gland in Guinea Pigs.

No. of animals	No. of electronarcoses	Epithelial height (mean of 100 follicles in μ)
5	0	5.54
4	10 (2 min)	8.15
1	11 (10 '')	9.54
1	7 (10 '')	8.30

In an earlier series of dog experiments in which electronarcosis was given for other purposes, the thyroids were removed and examined microhistometrically. Two of the dogs had been subjected to a prolonged series of electronarcoses prior to the acute experiment, 2 had had but a single previous electronarcosis, and the other 3 had not had any electronarcoses. No significant difference in the thickness of the thyroid epithelial cells existed between the control animals and those which had received a single previous electronarcosis. Those which had been subjected to a prolonged series of electronarcoses, however, showed a slight but significant thickening of the follicular cells.

Effect of Electronarcosis on the Concentration of Thyrotropic Hormone in Blood Serum.

‡ In 2 animals a complete motor paralysis of the hind legs developed following a series of electronarcoses. Postmortem examination disclosed, however, that this condition was due to a trauma of the spinal cord resulting from compression by the vertebræ during the strong extensor spasm which occurred during the initial stages of electronarcosis. Both of these accidents occurred when unusually high initial currents were used.

§ The authors wish to express their thanks to Miss Ruth E. Estey for her assistance in making the histological preparations.

TABLE II.
Influence of a Series of Electronarcoses on Concentration of Thyrotropic Hormone in Dog Plasma as Indicated by Injecting Groups of 10 or More Chicks with the Plasma.

			Chick thyroids			
			Weight in mg		Epithelial height in μ	
Dog		No. of electronarcoses	Control	Injected	Control	Injected
1	Before	0	3.3	3.3	3.6	3.6
2		0	2.9	2.9	3.7	3.6
3		0	3.8	3.5	2.6	2.9
4		0	3.8	3.7	2.6	2.8
Mean before electronarcosis			3.3	3.4	3.1	3.2
1	After	7	3.2	5.0	3.6	5.7
2		5	2.3	3.6	3.3	5.0
3		7	2.4	3.1	3.0	3.7
4		7	2.4	2.9	3.0	3.9
Mean after electronarcoses			2.6	3.7	3.5	4.6

Four dogs were subjected to electronarcosis once daily. In 3 of the animals 7 electronarcoses were given, in the other, 5. The duration of each treatment was 8 to 10 min. Just before the first, and 24 hours after the last treatment blood was collected from the dog. It was immediately centrifuged to separate the plasma from the cells. The plasma thus obtained was assayed for thyrotropic activity by injecting 0.2 ml of plasma into day-old cockerels daily for 4 days. The chicks were sacrificed on the 5th day and the thyroids removed. The weights of the chick thyroids and the microhistometric analyses of these glands from the post-electronarcotic sera have been tabulated in Table II, together with those of the uninjected control chicks.

The prenarcotic plasma of all 4 dogs showed no thyrotropic activity whatsoever. The mean weights of the thyroid glands of the injected chicks were the same as those of the uninjected controls, and the mean thickness of the acinar epithelium was the same in both groups. Following a series of electronarcoses, however, the dog serum produced a small but definite increase in gland weight and in epithelial height in the injected chicks.

Blood taken from one of the dogs (No. 2) one week after the last electronarcosis showed a reduced thyrotropic activity, and after 2 weeks no such activity was present.

The post-electronarcotic plasma of 3 of the 4 dogs also produced a moderate hypertrophy of the testes of the chicks used for the thyrotropic hormone assay, indicating that not only the thyrotropic but other endocrinotropic factors may be found in the blood in greater concentrations following the passage of electronarcotizing currents.

Conclusions. Experimental evidence indicates that a series of electronarcoses in animals results in hypertrophy of the thyroid, adrenals, and gonads. Since in the blood of electronarcotized dogs an increase in thyrotropic substance is found, it is believed that the mechanism by which this thyroid hypertrophy is obtained is by way of an increased secretion of the pituitary hormones. It might be conceivable that the general cortical and subcortical stimulation which results from passage of current through the brain could produce a direct nervous stimulation of the respective endocrine organs. However, whereas direct stimulation of certain endocrine glands is possible,[6] the response to such nervous stimulation is of a much smaller order than that resulting from activation of the organ by the specific hypophyseal "trophic" hormone. That direct stimulation of the thyroid is most likely not involved in the hypertrophic response to electronarcosis is shown by the increased thyrotropic activity imparted to dog serum by subjecting the dog to a series of electronarcoses. The increased endocrinotropic activity seems to be only temporary.

14882

Effect of Vitamins on Acetylcholine Synthesis. The Apparently Specific Action of Vitamin E.*

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In search of substances that modify the synthesis of acetylcholine the effect of vitamins on the acetylcholine synthesis *in vitro* was investigated.

Method. The synthesis of acetylcholine was studied by the method of Quastel, Tennenbaum, and Wheatley[1] with minor modifications.[2] Varying amounts of the substances were added to mixtures containing 100 mg minced fresh frog brain, 3 mg physostigmine salicylate, 4.8 mg glucose, and 3 cc Ringer's solution. The pH of the mixtures was adjusted to 7.4. Identical mixtures without the substances served as controls for the water soluble substances and mixtures containing an adequate amount of sesame oil for the oil-soluble substances. The mixtures were shaken and incubated aerobically for 4 hours at 37° C. After incubation the amounts of total acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog. The amount of acetylcholine synthesized was calculated by subtracting from the acetylcholine content of the incubated mixtures the acetylcholine content of identical nonincubated mixtures. By adding the substances in varying concentrations to incubated control mixtures after incubation it was ascertained whether the substances modified the sensitivity of the rectus abdominis muscle to the acetylcholine content of the mixtures during the 2 minutes of immersion for the biological assay. If so the changes were taken in account by the calculation.

Results. The amounts of acetylcholine synthesized in the presence of the substances used are given in Table I. A marked decrease of synthesis was found in the presence of low and increasing concentrations of vitamin A and K,[3] and high concentrations of B_1[4] and D.[5] A marked increase of synthesis was found in the presence of low and increasing concentrations of *a*-tocopherol, and of high concentrations of some of the members of the vitamin B group and vitamin C.

Discussion. An adequate explanation of the mechanism through which the substances used modify the synthesis of acetylcholine cannot yet be offered. Most of the substances used have a positive oxidation-reduction potential, and vitamin K may modify the activity of —SH containing substances, an active group probably contained in the enzyme involved in the synthesis of acetylcholine.[6]

The decrease of synthesis of acetylcholine in the presence of vitamin A may be significant in the body since stimulation of the sympathetic nervous system or injection of epinephrine[7–10] are known to be followed by an increased concentration of vitamin A in the serum because of an increased mobilization from its stores.

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TABLE I.
Effect of Vitamins on Synthesis of Acetylcholine.

	Amt of total acetylcholine synthesized in % of control*					
	Amts of the substances added to 100 mg frog brain (mg)					
Substance	3	0.3	0.03	0.003	0.0003	0.00003	0.000003
Vit. A†		12	34	67	81	90	99
Thiamine chloride		46	64	87	118		
Riboflavine	136	126	101	100	101		
Nicotinic acid	145	144	119	98	111		
Nicotinamide	125	120	110	107	106		
Calcium pantothenate	148	137	123	99	100		
p-Amino benzoic acid	123	110	104	104	105		
Pyridoxine	123	115	104	101	100		
Vit. C	140	119	98	96	97		
Vit. D‡		30	75	97	101	103	99
α-Tocopherol (SMACO)			225	185	162	130	110
Vit. K (menadione)		28	66	74	83		

* The S.E. of the mean for each value was less than $\pm 5\%$. Each value represents the average of 8 separate experiments. The amount of acetylcholine synthesized in μg per 100 mg frog brain followed by the S.E. of the mean was 1.50 ± 0.044.

† Each mg contained 500 I.U. vitamin A.

‡ Each mg contained 400 I.U. vitamin D.

α-tocopherol even in minute concentrations induced a striking increase of the synthesis of acetylcholine, suggesting that it has a specific action on this process. It is known that vitamin E and α-tocopherol modify the metabolism of lipids, phospholipids,[11-15] and may enhance phosphorylation.[11, 16-20] These processes may contribute to the increase of the synthesis of acetylcholine since choline occurs in the body as a constituent of phospholipids, and the energy required for the synthesis of acetylcholine may be supplied by energy rich phosphate bonds. However, it is possible that vitamin E is a part of the coenzyme involved in the synthesis of acetylcholine.

A close relationship between vitamin E and synthesis of acetylcholine in the body is suggested by the consideration that the placenta, an organ without nerve supply but rich in acetylcholine,[21] is also rich in vitamin E;[19] vitamin E deficiency is followed by a decrease of choline esterase content of tissues,[22, 23] a decrease known to occur in denervated muscles;[24,23] and deficiency of vitamin E may result in the degeneration of the end-plates in striated muscle.[25]

Summary. 1. The effect of vitamins on the synthesis of acetylcholine was investigated. 2. Vitamin A and K decreased the synthesis of acetylcholine in low and increasing concentrations. Vitamin D did not modify the synthesis in low concentrations and decreased it in higher ones. Vitamin B_1 slightly increased the synthesis of acetylcholine in low concentrations and decreased it in higher ones. 3. Ribo-

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flavin, nicotinic acid, nicotinamide, calcium pantothenate, p-amino benzoic acid, pyridoxine, and vitamin C did not modify the synthesis in low concentrations and increased it in higher ones. 4. Vitamin E increased the synthesis in low and increasing concentrations.

14883

Phlebostatic Axis and Phlebostatic Level, Reference Levels for Venous Pressure Measurements in Man.*

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In the determination of venous pressure both in the clinic and in the laboratory it is often necessary to make the measurements with the subjects in positions (sitting, intermediate sitting, with parts under study in many positions relative to the level of the heart) other than supine. The levels of reference available at the present time fail to fulfill the requirements of practicability and accuracy necessary for clinical and experimental studies. At least 9 different reference levels have been given for the measurement of venous pressure.[1-8] Some have been related to anatomic parts of the body and some to the horizon or examination table. The lack of consistency in the reference or heart levels makes it difficult to undertake studies on venous pressure. The present studies were conducted to find a point of reference for venous pressure measurements which may be applicable to subjects of any build, in many positions, and with the vein under study in any positions necessary for the study of clinical problems concerned with venous pressure measurements in any superficial vein.

Method and Materials. The apparatus, the Phlebomanometer described elsewhere, is accurate to ± 1 mm of water, and checked satisfactorily at frequent intervals with a water manometer.[9] Approximately 265 determinations were made on 165 normal young adults (ages 16-34 years) of both sexes and negro and white races who rested prior to and during measurements on a firm plywood table with an adjustable head. The veins studied are indicated below.

Results. In 99 subjects of both races and sexes it was found that when a plane passing longitudinally through the body parallel to its anterior surface and midway between the dorsal surface of the thorax and the base of the xiphoid process was used as the reference level the venous pressure measurements in the median basilic veins varied relatively little even though the thickness of the chests varied considerably (78-135 mm, mean 97 mm). That the plane described is a good reference level was further substantiated by the fact that in 10 selected subjects with very thick chests (mean reference level 126 mm from dorsum of trunk) and 10 with very thin chests (mean reference level 89 mm from dorsum of trunk) the venous pressure

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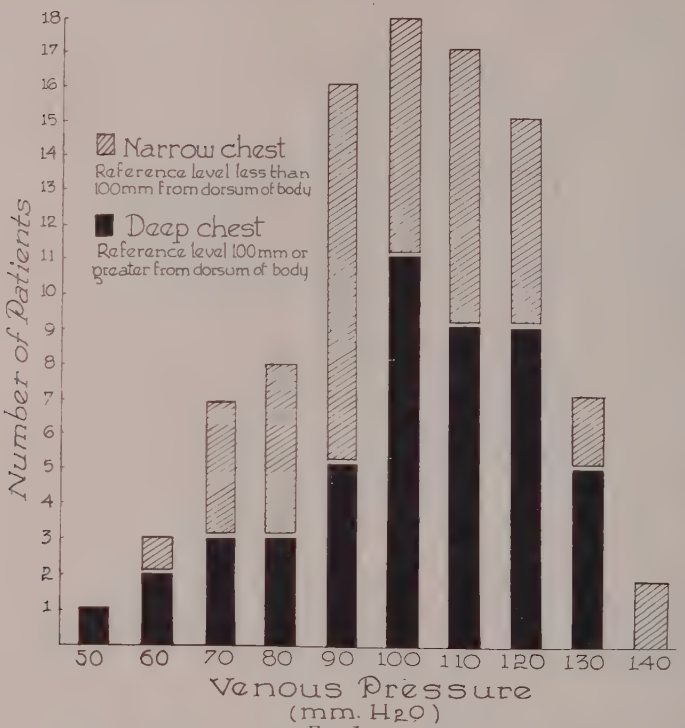

FIG. 1.

Distribution curve of venous pressure measured at the phlebostatic axis (see text) in 99 normal subjects with the axis less than 100 mm from the dorsal surface of the trunk and also in subjects with the axis 100 mm or more from the dorsal surface of the trunk. The ages varied from 16-34 years and included both sexes and the white and negro races. The number of subjects in each venous pressure group may be determined by projecting the length of the columns on the ordinate. For example, the subjects with venous pressures of 70 mm of water consisted of 2 with thick chests and 1 with a narrow chest; of the subjects having 100 mm of water venous pressure there were 5 with thick chests and 11 with thin chests. It can be seen that the distribution of venous pressures was essentially the same for the subjects with thin and thick chests when the phlebostatic axis was used as the level of reference.

measured at the above reference plane averaged 104 and 103 mm of water respectively. Furthermore, when a distribution curve was drawn to correlate venous pressure and chest thickness no significant differences were found in the venous pressure values for chest thickness provided the above reference plane for heart level was used (Fig. 1).

To determine the plane of reference, heart level, for subjects in the erect sitting position the arm was elevated until the venous pressure in the basilic vein reached that previously recorded for the supine position. The level of the vein in relation to the intercostal space was noted. From Fig. 2 it can be seen that in the majority of instances the plane passed through the fourth intercostal space at its junction with the sternum.

The frontal plane described for the supine position and the cross sectional plane for the erect position intersected to form a transverse axis which passed through the thorax from side to side midway between the anteroposterior surfaces at the level of the junction of the fourth intercostal space with the margin of the sternum. In order to determine whether or not this axis can be used as a *universal axis* of reference or heart level for venous pressure measurements with the subject in positions between supine and erect sitting the following studies were conducted:

1. The venous pressures were recorded in a

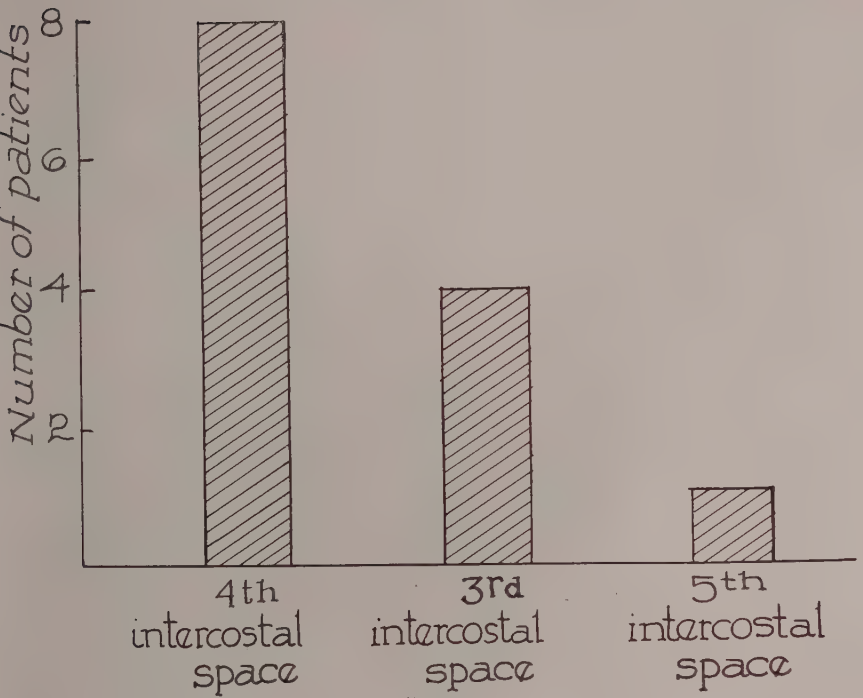

FIG. 2.

In 13 normal young adults the venous pressure in the basilic vein was determined with the subject in the supine position (see text for method). The subjects then sat erect and the vein was elevated until the pressure in the basilic vein reached that recorded in the supine position. In the majority the vein was found to have been raised to the level of the junction of the fourth intercostal space with the lateral margin of the sternum. There were, however, some variations.

dorsal vein of the hand with the subjects in the supine position and in intermediate sitting positions in which the dorsum of the trunk and examining table formed angles of 0, 25, 35, 45, 55, and 90 degrees respectively. Venous pressure measurements were made in each of these positions with the vein under study in the same horizontal plane as the transverse axis of reference. The venous pressure values obtained were essentially the same regardless of the position of the trunk (Fig. 3).

2. Further to test this phenomenon the vein on the dorsum of the hand was kept at the reference level for the supine position and the trunk was flexed to the sitting positions described above. For each position the venous pressure in a dorsal vein of the fixed hand was measured and found to increase by a pressure equal to a column of water extending from the vein of the hand to the new position of the transverse axis of reference.

3. In a third group of studies the trunk was flexed into the above sitting positions and then the hand was elevated until the pressure in a dorsal vein of the hand reached that previously determined for the supine position. Invariably the vein on the dorsum of the hand was found to be at the horizontal level passing through the transverse axis.

A study of widely separated veins important in the evaluation of clinical states concerned with vascular disease and particularly venous obstruction (superficial inferior epigastric, superficial superior epigastric, paraumbilical, cephalic, saphenous, dorsal veins of the feet, thoracoepigastric) indicated that the transverse axis proved to be consistently applicable as a site from which to measure venous pressure.

Comment. The literature shows that most

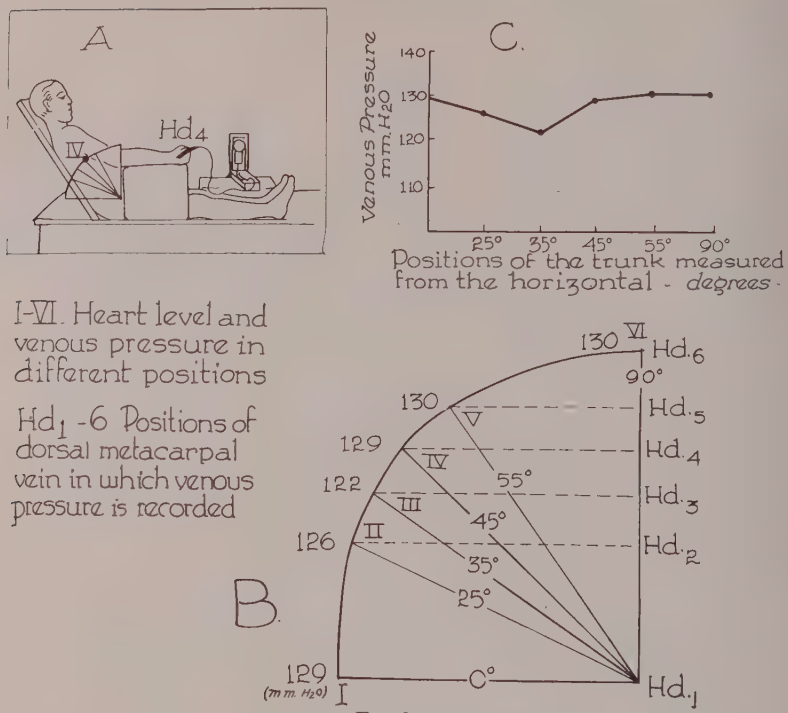

FIG. 3.

The venous pressure in a dorsal vein of the hand was recorded in 10 subjects in various sitting positions varying from horizontal to erect using the phlebostatic axis as the level of reference in all positions. Insert A shows diagrammatically how the trunk was flexed and the hand elevated to the phlebostatic level in the 6 positions indicated. Insert B shows the relative positions of the hand and phlebostatic axis for various intermediate sitting positions. Insert C represents graphically the venous pressure in mm of water for the 6 positions of the trunk. It can be seen that with the use of the phlebostatic axis as the level of reference the 6 positions of sitting did not alter the venous pressure in the dorsal vein of the hand.

observers have employed different reference points for heart level for different positions of the body.[10] Others have limited their studies to one position and employed a reference point suitable only for the one position of study.[11-12] It is obvious that such methods have marked clinical limitations. Furthermore, many observers have tried to determine heart level on an anatomic basis without making a serious effort to correlate the anatomic level with physiologic venous phenomena. Although an anatomic landmark is necessary, the validity of this landmark must be based upon physiologic data. In the observations reported above, it was made certain that the anatomic landmark employed to define the universal axis of heart level correlated and conformed to physiologic venous phenomena. Obviously no one landmark will satisfy absolutely all subjects but the transverse axis described above appears to lead to relatively little variations in results when normal subjects are studied. From roentgenographic studies of living subjects, the transverse axis passes through the openings of the venæ cavæ into the right auricle. When studying venous pressures in any vein with the subject in any position described but always with the transverse axis parallel to the horizon, it is only necessary to refer the vertical level of the vein to the horizontal plane passing through the trans-

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11 Ferris, E. B., Jr., and Wilkins, R. W., *Am. Heart J.*, 1937, **13**, 431.

12 Berger, A. R., *Am. Heart J.*, 1937, **13**, 440.

verse axis.

It is proposed to call this transverse axis of heart level the *phlebostatic axis* and any of the horizontal planes passing through this axis used as the reference level the *phlebostatic level* in order to avoid confusion with the many different "heart level" landmarks described previously in the literature.

Summary. It has been found that the reference level or heart level for the measurement of venous pressure is an axis which runs transversely through the thorax at the point of junction of a plane passing cross-sectionally through the fourth intercostal space adjacent to the sternum with a frontal plane passing midway between the posterior surface of the body and the base of the xiphoid process of the sternum. Horizontal planes passing through this axis are the reference levels or heart levels to be used for that particular position of the patient. It is proposed to call the axis the *phlebostatic axis* and the horizontal planes passing through the axis the *phlebostatic level.*

14884

Biological Activity of N-Methylnicotinamide and Nipecotic Acid.*

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The use of microbiological vitamin assays always involves an uncertainty in that the activity of certain related compounds may be different for the test organism than for the animal. In the case of nicotinic acid, no derivative has been found, to our knowledge, which is active for *L. arabinosus* but inactive when ingested by an animal. In fact, it has been established that a number of precursor compounds readily elicit an active response from the animal, but must first be hyrolyzed to be active for *L. arabinosus.*[1-3] It is now generally accepted that autoclaving samples with 1N alkali gives a degree of potency with *L. arabinosus* which agrees closely with the biological vitamin activity for animals. The results given in two recent reports may raise some question about this assumption.

Von Euler *et al.*[4] have reported that hydrogenated nicotinic acid (nipecotic acid) is active for *Staph. aureus* and *Proteus vulgaris.* This is of interest because nipecotic acid has been found inactive for the dog[5] and dysentery bacilli.[6] Najjar *et al.*[7] have reported that N-methylnicotinamide chloride (nicotinamide methochloride) is active in preventing and curing nicotinic acid deficiency in dogs and produces a growth response with *E. coli.* This compound has previously been found inactive for the dog,[5] and *L. arabinosus.*[3] Furthermore, many investigators have tested trigonelline, which is chemically related to N-methylnicotinamide as nicotinic acid is to nicotinamide, and have found it inactive for the dog, man and all microörganisms tested.[1]

In this paper, we wish to present further studies on the activity of N-methylnicotin-

*Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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5 Woolley, D. W., Strong, F. M., Madden, R. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1938, **124**, 715.

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7 Najjar, V. A., Hammond, M. M., English, M. A., Worden, M. B., and Deal, C. C., *Bull. Johns Hopkins Hosp.*, 1944, **74**, 406.

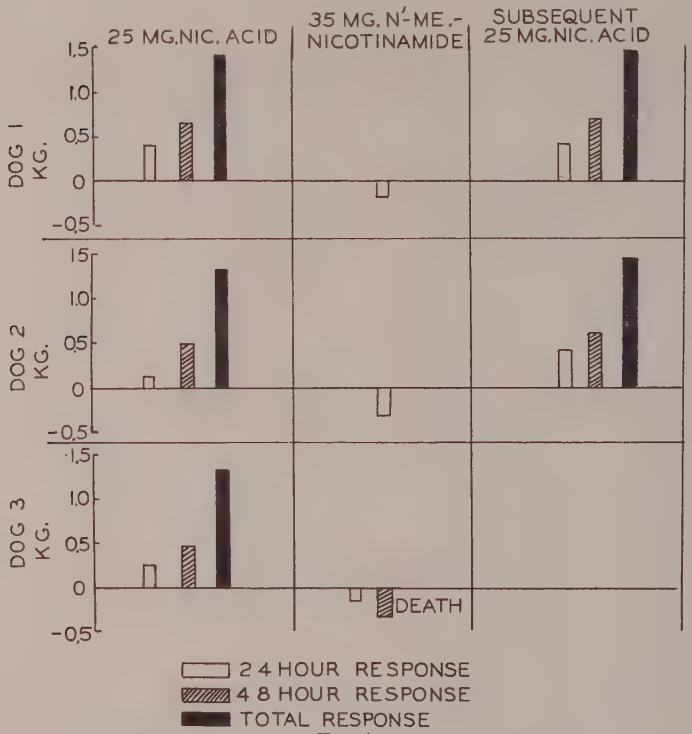


FIG. 1.
Weight response of blacktongue dogs to nicotinic acid and N'-Me-Nicotinamide.

amide and nipecotic acid.

Experimental. Nipecotic acid was tested with *L. arabinosus* and found to be only 1/10,000 as active as nicotinic acid. The procedure used has been described in detail[8] and acid production was measured after 48 hours incubation. The very slight but definite activity was probably due to impurities.

With the improved synthetic ration containing "folic acid,"[9] N-methylnicotinamide chloride was tested for its activity in the dog. It has been the experience of this laboratory that in an individual dog on this diet, nicotinic acid deficiency may be repeatedly produced and cured, with uniformly rapid responses to nicotinic acid administration. Three animals were standardized by producing nicotinic acid deficiency and feeding a sub-optimum dose of 25 mg of nicotinic acid. The dogs were again brought down with a severe nicotinic acid deficiency and nicotinamide methochloride, equivalent to 25 mg nicotinic acid on the molar basis, was administered orally. The dogs continued to lose weight during the next 24 hours. Twenty-five mg nicotinic acid were then fed and the dogs began eating and drinking within 12 hours, and made a weight gain which checked very well with the response in the standardization. This indicates that there was no supplemental action due to N-methylnicotinamide. In the case of the third dog, no further supplement was given for 48 hours after the nicotinamide methochloride administration. At this point the animal was in extremely poor condition and died before a cure could be effected with nicotinic acid. The results are presented graphically in Fig. 1.

ssion. It should be pointed out that in

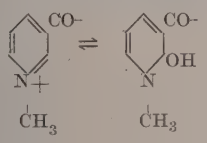
[8] Krehl, W. A., Strong, F. M., and Elvehjem, C. A., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 471.

[9] Krehl, W. A., and Elvehjem, C. A., *J. Biol. Chem.*, in press.

Najjar's experiments, high levels of nicotinamide methochloride were employed. In the case of the *E. coli* tests, 20γ-100γ/cc of medium were used. These amounts seem very high in view of the nicotinic acid requirements of other bacteria that have been studied, *e. g.*, 0.1γ of nicotinic acid/cc produces maximum growth with *L. arabinosus*. It has been our experience that almost any preparation of a compound chemically related to nicotinic acid contains enough impurities to show activity if it is added to bacterial media at very high levels.

The amounts of nicotinamide methochloride administered to dogs by Najjar *et al.* were also very high. In their prophylactic experiments, 50 mg and 25 mg were fed per day, which is in great excess of the nicotinic acid requirement. In the curative treatments, 250 mg were given within 12 hours and 50 mg per day thereafter, which also greatly exceeds the amount of nicotinic acid required to cure black-tongue. We have consistently obtained cures with 25-50 mg of nicotinic acid.

We have no proof that N-methylnicotinamide is completely devoid of biological activity, but since we have used levels of this compound which are comparable to amounts of nicotinic acid that have been found effective, we are convinced that if it is biologically active, its potency must be extremely low. Najjar *et al.* suggest that the following structure is essential for biological activity



regardless of the radical that is attached to the carboxy group. If this theory is correct, trigonelline and N-methylnicotinamide should be at least as active, if not more active, than nicotinic acid. N-methylnicotinamide has already been discussed. In the case of trigonelline, there is overwhelming evidence that it has no biological activity whatsoever. Finally, von Euler *et al.*[4] have found guvacin (1-2-5-6 tetrahydro nicotinic acid) active for *Staph. aureus* and *Proteus vulgaris*. When this compound was converted to the N-methyl derivative (arecaidine), there was no activity whatsoever.

Najjar is of the opinion that the correlation between N-methylnicotinamide excretion and nicotinic acid deficiency supports his view. It seems more likely, however, that methylation is merely the usual treatment of pyridine-type compounds in the body, since pyridine itself is methylated.[10] It is entirely logical that with low nicotinic acid intake, the excretion of N-methyl derivatives would drop more or less proportionately.

Summary. 1. Nipecotic acid was found to be only 0.01% as active as nicotinic acid for *L. arabinosus*. 2. N-methylnicotinamide chloride was found to be ineffective in curing black-tongue in dogs. 3. The theory of Najjar *et al.* as to the relation of chemical structure to anti-blacktongue activity is discussed.

[10] Harrow, B., *Textbook of Biochemistry*, 3rd Ed., W. B. Saunders Co., 1943, p. 252.

14885

Occurrence of Hyaluronidase and Lecithinase in Relation to Virulence in *Clostridium welchii.*

E. H. Kass, H. C. Lichstein and B. A. Waisbren. (Introduced by C. H. Bunting.)

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Recent studies of the pathogenesis of the gas-gangrenous infection have focused attention on the role of the enzyme hyaluronidase in influencing the spreading character of the lesion. This enzyme has been isolated from such diverse sources as testicular extract,[1,2] spleen,[3] snake and spider venoms,[4] and extracts of various tumors.[5] It is also produced by many bacteria, including pneumococci[6] and certain strains of non-mucoid hemolytic streptococci,[3,7] and has been demonstrated in cultures of *Cl. welchii, Cl. septicum,* and *Cl. oedematiens.*[8–10] Hyaluronidase has been identified as one of the most active spreading factors;[1,11,12] it acts by hydrolyzing hyaluronic acid, a polysaccharide which is found in the interfibrillar substance of skin,[13] as well as in synovial fluid,[14] vitreous humor,[15] umbilical cords,[15] certain tumors,[5,16,17] and capsules of streptococci of Groups A and C.[18–20]

Although there is widespread acceptance of the thesis that the spreading nature of the *Cl. welchii* infection is due to the presence of hyaluronidase,[11] there are few actual studies of the occurrence of the enzyme in cultures of that organism. McClean and his coworkers[10] found that in 32 strains of *Cl. welchii* which they studied, 12 produced hyaluronidase, and 11 of these were toxicogenic strains, *i.e.* showed lecithinase activity. Of the 20 strains which failed to produce hyaluronidase, 11 were toxicogenic. It is of interest to note that of 20 strains isolated from cases of gas gangrene, 18 produced hyaluronidase.

Since an evaluation of the role of hyaluronidase in the virulence of *Cl. welchii* seemed necessary, the following study was undertaken in order to relate the occurrence of hyaluronidase in freshly isolated strains of *Cl. welchii* to virulence and to the capacity for producing lecithinase. The latter determinations were made on the apparently well-established assumption that lecithinase activity is a measure of concentration of alpha toxin in these organisms.

Methods. Ninety-four strains were isolated, 40 of these from human feces, 24 from soil, and 30 from animal feces. Samples of the source material were heated to 80°C for 10 minutes in skimmed milk containing reduced iron.

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4 Duran-Reynals, F., *J. Exp. Med.,* 1939, **69**, 69.

5 Pirie, A., *Brit. J. Exp. Path.,* 1942, **23**, 20.

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13 Meyer, K., and Chaffee, E., *J. Biol. Chem.,* 1941, **138**, 491.

14 Meyer, K., Smyth, E., and Dawson, M. H., *J. Biol. Chem.,* 1939, **128**, 319.

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TABLE I.
Hyaluronidase- and Lecithinase-activity, and virulence of strains of *Clostridium welchii*.

Hyaluronidase	Lecithinase	No. Strains	No. Virulent*	No. Toxic†
+	+	32	18	12
—	+	34	16	8
+	—	17	4	10
—	—	11	3	2

*Virulent strains: Those which killed mice with typical local lesions after intramuscular injection of 0.2 ml of broth culture.

†Toxic strains: Those which are not virulent but which killed mice if 0.8 ml of broth culture were given intra-abdominally along with intramuscular injections.

After overnight incubation, tubes showing stormy fermentation were streaked on tryptose agar plates containing 0.025% sodium azide[21] and 5% defibrinated rabbits' or sheep's blood. These were incubated anaerobically for 48 hours at 37°C, after which the typical, well-isolated, hemolytic, mucoid colonies of *Cl. welchii* were picked and transferred to milk containing iron. After overnight incubation, carefully chosen colonies invariably produced typical stormy fermentation. Transfers were then made from the clotted milk to tubes of infusion broth containing cooked meat and, after overnight incubation, these cultures were examined and stored in the refrigerator.

We are not aware of the previous use of sodium azide for the purpose of facilitating the often difficult task of isolating a pure culture of *Cl. welchii*. In media in which the azide was not used, the number of successful isolations was greatly diminished, while plates containing the azide never failed to give well-isolated colonies free of the usual difficulties with spreading contaminants.

For the determinations of hyaluronidase, lecithinase, and mouse virulence, the stock cultures were transferred to another tube of cooked meat medium and also to a tube of plain broth containing 0.25% hyaluronic acid purified from human umbilical cords. The usual difficulties and losses attending the filtration of the highly viscous hyaluronic acid for the purpose of sterilization may be obviated by weighing the dried polysaccharide into a sterile container and allowing the material to soak in 95% ethyl alcohol for a few days. The alcohol may then be carefully evaporated off, and sterile saline or broth added to dissolve the polysaccharide with no loss of material. All tests were performed within a week after the start of the isolation procedure.

Hyaluronidase activity was determined in two ways. The more important and more accurate method was to use the tube containing hyaluronic acid as a substrate. Eighteen-hour cultures in this medium were neutralized to phenol red and centrifugated. The supernate was mixed with acidified horse serum in the presence of acetate buffer at pH 4.2, as in the methods of Seastone,[22] and Kass and Seastone.[23] Since hyaluronic acid usually produces heavy turbidity under these conditions, failure of the turbidity to appear is an indication of hyaluronidase activity. The results could usually be anticipated following the addition of phenol red, since cultures producing hyaluronidase will hydrolyze hyaluronic acid to fermentable end-products with consequent acid production; tubes in which there had been no breakdown showed little change in reaction.

Hyaluronidase was also sought in supernates of centrifugated meat broth cultures by mixing equal amounts of the supernate, dilute hyaluronic acid solution, and phosphate buffer at pH 7.0. These were incubated overnight in the presence of a few drops of toluene and examined by overlaying the mixture on acidified horse serum in precipitin tubes.[22] Since the concentration of hyaluronic acid had previously been adjusted to give a distinct ring when laid over acidified horse serum, failure of any precipitate to appear after incubation with the culture supernate indicated the presence of hyaluronidase in the culture.

[21] Lichstein, H. C., and Soule, M. H., *J. Bact.*, 1944, **47**, 221.

[22] Seastone, C. V., *J. Exp. Med.*, 1943, **77**, 21.

[23] Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **79**, 319.

Lecithinase was detected by a modification of the van Heyningen method:[24] one volume of egg-yolk substrate was mixed with an equal volume of saline and one-half volume of culture supernate. Control tubes were simultaneously set up containing 10-15 units of antitoxin instead of saline. These were read after a few hours' incubation at room temperature.

Mouse virulence was determined by injecting 0.2 ml of the meat broth cultures into the thigh muscles of unprotected mice and also into mice previously protected by the intraabdominal injection of 15 units of antitoxin. The antitoxin which was available to us was active against *Cl. septicum* toxin as well as toward *Cl. welchii* toxin. For this reason, care was taken to use only strains which produced typical mucoid colonies on blood agar and rapid stormy fermentation in milk. Virulent strains produced typical gas-containing lesions and killed unprotected animals within 24 hours. Strains which failed to kill by this procedure were passed through mice by injecting 0.2 ml intramuscularly and 0.8 ml intraabdominally. The heart's blood of animals dying within 24 hours was cultured and passed again through mice by the intramuscular route only. Fourteen strains proved virulent following this procedure, after they had failed to kill in the original test. These are recorded in the data as virulent strains along with those strains which were lethal upon first trial.

The desirability of using freshly isolated strains in studies of this type is illustrated by the observation that certain strains of *Cl. welchii* which were obtained from Dr. Elizabeth McCoy produced hyaluronidase in meat media when tested in 1942 by one of us (E.H.K.) working with Dr. C. V. Seastone, but failed to produce the enzyme when tested recently even when grown in media containing hyaluronic acid. It should also be noted that some of our strains which were originally virulent for mice lost this property after storage in the refrigerator for as little as 6 weeks.

Results. The results, presented in Table I, demonstrate that while the precise role of hyaluronidase in the gas gangrenous lesion must await the completion of histological studies, the enzyme as such is not directly linked with virulence in the strains of *Cl. welchii* studied. Thus, 46% of the virulent strains failed to produce hyaluronidase, whereas only 17% failed to produce lecithinase. Of all the strains examined, the group producing both hyaluronidase and lecithinase showed the greatest number of virulent strains (56%), although almost as many (47%) of the strains in the group producing lecithinase alone were virulent to the mouse.

Evans[25] has recently reported the failure of sera with high anti-hyaluronidase activity to be effective in protecting against infection with *Cl. welchii,* and has pointed out that the protective capacity of antitoxic sera is related to their anti-lecithinase activity.

If a less rigorous test for virulence is applied, namely, the ability of a strain to kill mice after intramuscular injection of 0.2 ml of an 18-hour broth culture along with intraabdominal injection of 0.8 ml of the culture (such strains are "toxic" strains in our designation), it is found that 90% of the hyaluronidase-producing strains and 82% of the lecithinase-producing strains are toxic. We have chosen the more rigorous method as the more significant because we feel that it indicates those strains which are capable of producing an active, typical lesion, rather than a generalized, rapidly occurring toxemia and peritonitis without much local involvement.

While the enzymes occurred in all combinations, including the absence of both, and virulent strains were encountered with each combination, there was no correlation between any combination of enzymes and source of the strain. However, the incidence of lecithinase is considerably greater than that of hyaluronidase; 70% of all strains produced lecithinase, whereas only 52% produced hyaluronidase. The occurrence of virulent strains producing neither enzyme is of great interest, and offers obvious lines for further investigation.

Thirteen of the 14 strains whose virulence was increased by mouse passage produced the same combinations of enzymes before and after such passage. The exception was a strain which produced lecithinase alone before pas-

[24] van Heyningen, W. E., *Biochem. J.*, 1941, **35**, 1246.

[25] Evans, D. G., *J. Path. and Bact.*, 1943, **55**, 427.

sage, but produced hyaluronidase as well after becoming more virulent. While this observation is recorded for its theoretical interest, there is no assurance that the strain recovered after mouse passage was the same strain that was injected, hence the validity of this observation must await confirmation from many sources.

The need for cultivating organisms in the presence of hyaluronic acid in order to demonstrate hyaluronidase is amply borne out by the observation that 49 of 54 strains producing hyaluronidase did so only when grown in media containing hyaluronic acid.

Summary. 1. The isolation of 94 strains of *Cl. welchii* from human feces, soil, and animal feces was facilitated by the use of sodium azide in the culture media. 2. These strains were tested for mouse virulence, and for ability to produce the enzymes lecithinase and hyaluronidase. Fixty-six per cent of the strains producing both enzymes were virulent, as were 47% of those producing only lecithinase. Twenty-four per cent of those strains producing only hyaluronidase and 28% of those producing neither enzyme were virulent. 3. Of 41 mouse virulent strains isolated, 83% produced lecithinase, and 54% produced hyaluronidase. Of all strains isolated, 70% produced lecithinase whereas only 52% produced hyaluronidase.

4. It is concluded that regardless of the role of hyaluronidase in a gangrenous lesion, its *in vitro* production by a given strain of *Cl. welchii* bears no necessary relationship to the virulence of that strain for mice.

14886

Inactivation of the Antibiotic Activity of Penicillin by Cysteine Hydrochloride. I. Chemical Aspects of Inactivation.

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It is well known that penicillin can be easily inactivated by numerous chemical agents, such as acids, alkalis, etc. Recently Atkinson and Stanley[1] reported the occurrence of "penicidin-suppressors" in a variety of materials, among which they listed thioglycolic acid. They were aware of the fact that the abolition of the antibiotic activity may be the result of either a specific chemical reaction or of an interference with an essential enzyme reaction in a manner similar to the inhibiting effect of p-aminobenzoic acid on sulfonamide bacteriostasis. In another communication,[2] they extended the study to various sulfhydryl compounds including cysteine as well as other reducing agents. Their results suggested to them "that the mechanism of this suppression is a chemical reaction between penicidin and the SH group."

Woodruff and Foster[3] studied the effect of sulfhydryl group (cysteine) on penicillinase, an enzyme which destroys penicillin. In one of their experiments, they used as a control a mixture of penicillin and cysteine with heat-inactivated penicillinase, and found, on incubation, a decrease in the penicillin content. Woodruff and Foster, however, did not extend their investigation of the phenomenon.

In October 1943, during the course of a study on the effect of different chemicals on the stability of penicillin, we observed that penicillin, in contact with neutralized cysteine[4] hydrochloride, rapidly loses its antibiotic activity. In the light of the experiments we are

[1] Atkinson, N., and Stanley, N. F., *Australian J. Exp. Biol. and Med. Science*, 1943, **21**, 249.

[2] Atkinson, N., and Stanley, N. F., *Australian J. Exp. Biol. and Med. Science*, 1943, **21**, 255.

[3] Woodruff, H. B., and Foster, J., *J. Bact.*, (in press).

[4] Cavallito, C. J., and Bailey, J. H., *Science*, 1944, **100**, 390.

TABLE I.
Destruction of the Antibiotic Activity of Crystalline Penicillin by Decreasing Amounts of Cysteine Hydrochloride.

Cysteine hydrochloride Mg/ml	μ Penicillin per ml	Percent Destruction 48 hr
2.5	20	99
1.25	"	99
0.625	"	99
0.313	"	99
0.157	"	99
0.0785	"	97
0.0393	"	50-70
0.000	"	0

The reaction was allowed to proceed at 37° for 48 hours at pH 6.5 in an atmosphere of nitrogen.

to report in this communication, such a loss in activity is probably due to the destructive effect of cysteine.

Experimental. A procedure as described below was used in the early experiments. One ml of a solution of crystalline penicillin* (0.333 mg per ml) was added to each of 2 test tubes containing 8.0 ml of a M/10 phosphate buffer 7.6. To one tube was then added 1.0 ml of a freshly prepared neutralized cysteine hydrochloride solution (10 mg per ml) and to the other was added 1.0 ml of a 0.90% sodium chloride solution. After incubation at 37° for 2 hours, the antibiotic activity was determined by making 2-fold serial dilutions using 0.5 ml amounts, adding constant amounts (0.5 ml of a 10^{-6} dilution) of a penicillin sensitive organism (*Staphylococcus aureus,* Heatley), and incubating at 37° for 16 hours. The potency was determined by comparison with a crystalline penicillin standard tested at the same time. It was found that less than 0.2% of the original activity remained in the penicillin-cysteine mixture whereas there was no appreciable loss of activity in the control. In other words, 10 mg of cysteine had destroyed approximately 550 Oxford units of penicillin. In experiments like the one described above, air was not excluded and a part of the cysteine was oxidized to cystine which has been isolated and identified. In order to minimize the loss of cysteine hydrochloride due to oxidation by air, the reaction in later experiments was allowed to proceed in an atmosphere of nitrogen. The results of such an experiment performed at pH 6.5 and 37° for 48 hours are given in Table I.

It can be seen from Table I that under the experimental conditions about 0.04 to 0.08 mg of cysteine hydrochloride were sufficient to inactivate 0.02 mg of crystalline penicillin. In another experiment in which the reaction extended over a period of only 5 hours (other conditions remaining the same) inactivation was reduced about 15-fold. Expressed in terms of Oxford units destroyed, approximately 30 units were destroyed by 0.08 mg of cysteine in 48 hours while in 5 hours 1.2 mg of cysteine were required to destroy the same amount of penicillin.

Our preliminary experiments indicated that the rate of inactivation was dependent on hydrogen ion concentration. Within the pH range tested (5.3 to 7.6), the rate of destruction increased with increasing alkalinity. Hence we believe that the inactivation of penicillin may be due to a chemical reaction. Since the inactivation may involve any one or more of the reactive groups (the amino-, carboxyl-, and sulfhydryl-groups) in the cysteine molecule, it is of interest to ascertain, if possible, how the reaction takes place. To this end we have tried but failed to inactivate penicillin by means of other amino acids, such as glycine, cystine, methionine, or serine (an analogue of cysteine, having an oxygen atom in place of the sulfur atom). A comparison between the chemical structure of these com-

*The crystalline sodium salt of penicillin, obtained first by MacPhillamy and Wintersteiner in July 1943 (cf. R. D. Coghill, *Ind. & Eng. Chem.*, News Edition, **22**, 593 (1944)) was kindly furnished by Dr. O. Wintersteiner of the Division of Organic Chemistry of this Institute.

TABLE II.
Inactivation of Penicillin by Thioglycolic Acid.

Thioglycolic Acid Mg	Penicillin Oxford Units	pH	Destruction %
200	40	6.4	95
50	40	6.6	30-50
12.5	40	6.7	0
3.1	40	6.8	0
0.0	40	6.5	0
200	40	7.6	95
50	40	7.8	88
12.5	40	8.0	50
0.0	40	7.8	0

The reaction was allowed to proceed at 37° for 48 hours.

pounds and cysteine suggests that a free sulfhydryl group is essential for the destruction of the antibiotic activity. If this hypothesis is correct, one might expect thioglycolic acid to be able to inactivate penicillin. The results of a typical inactivation experiment with thioglycolic acid at different pH's are given in Table II.

Thus thioglycolic acid is a far less potent inactivating agent than cysteine. As a matter of fact, we found that simple mercaptans will not inactivate penicillin. These results, therefore, indicate that the inactivation of penicillin by cysteine involves more than the sulfhydryl group.

Summary. The antibiotic activity of penicillin can be easily abolished by cysteine in a slightly alkaline solution. The rate of inactivation is dependent on the amount of cysteine and pH. In an atmosphere of nitrogen, one mg of penicillin can be inactivated by several mg of cysteine at pH 7.8, but to a lesser extent with thioglycolic acid. Inactivation of penicillin does not take place if other amino acids, *e. g.* cystine, methionine, serine, or glycine are used. Hence we believe that the inactivation of penicillin by cysteine is a chemical reaction and that it may involve both sulfhydryl and amino groups of cysteine.

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Seasonal Fluctuations in Susceptibility of Guinea Pigs to Experimental Cavian Poliomyelitis.*

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Following the adaptation of SK poliomyelitis virus from monkey to mouse through intermediate cotton rat passage, further transmission of the murine strain to guinea pigs has been described.[1,2] A fixed strain of cavian virus was finally obtained which has now been carried over more than 70 serial passages. The disease thus produced in guinea pigs is characterized by the occurrence of flaccid paralysis, and the lesions in the central nervous system of paralyzed animals are "remarkably like those of poliomyelitis in monkey and man."[3] Serological identity of the murine and cavian strain was established by cross neutralization tests in mice and in guinea pigs. As far as any relationship between simian and cavian polio-

* Aided by grants from the Dr. Philip Hanson Hiss, Jr., Memorial Fund, the Warner Institute for Therapeutic Research, and anonymous donors.

1 Jungeblut, C. W. and Sanders, M., *J. Am. Med. Assn.*, 1941, **116**, 2136.

2 Jungeblut, C. W., Feiner, R. R. and Sanders, M., *J. Exp. Med.*, 1942, **76**, 31.

3 Wolf, A., *J. Exp. Med.*, 1942, **76**, 53.

TABLE I.
Incidence of Paralysis in Guinea Pigs Following Intracerebral Injection with Cavian or Murine Virus at Different Times of the Year.

Dates of Injection	Cavian Virus (Current Harvest)		Murine Virus: Current Harvest		Murine Virus: Winter Harvest*		Murine Virus: Summer Harvest†	
1/42	7/8		5/8					
2	10/10	25/26=96%	3/4	11/16=69%				
3	8/8		3/4					
4	3/4		3/3					
5	4/4		6/8					
6	4/4		1/4		2/4			
7	4/4	14/14=100%	2/8	11/27=41%	4/8	11/24=46%		
8	4/4		5/11		1/4			
9	2/2		3/4		4/8			
10	2/4		2/4		2/4			
11								
12			2/2				3/4	
1/43	3/4	6/8=75%	2/3	10/13=77%				14/28=50%
2	3/4		3/4				10/23	
3			3/4				1/1	
4								
5	4/7		3/4					
6			3/3		3/3			
7	2/4	6/8=75%	0/3	4/10=40%	1/4	4/10=40%		
8	4/4		1/4		0/3			
9								
10								
11								
12	2/2						8/12=67%	
Totals	66/77=86%		50/85=59%		17/38=45%		22/40=55%	

Numerator = No. of paralyzed guinea pigs; denominator = No. of injected guinea pigs.
* Feb. '42 harvest tested June-Sept. '42; Feb. '43 harvest tested June-Aug. '43.
† June-Aug. '42 harvest tested Dec. '42-Mar. '43; June-July '43 harvest tested Dec. '43.

myelitis virus is concerned, the available evidence rests on the following observations: (1) similarity in the symptomatology and pathology of the two infections, (2) limited but definite pathogenicity of the cavian virus for rhesus monkeys, (3) inability to recover injected cavian virus from the tissues of monkeys previously paralyzed by simian virus, and (4) inactivation *in vitro* of cavian virus by antiserum of a horse hyperimmunized with simian virus. Confirmatory evidence that the simian SK virus can be acclimated from cotton rat to guinea pigs with the production of paralysis, has been adduced by Toomey and Takacs.[4] More recently, another strain of human poliomyelitis virus, *i. e.* MM, has been adapted from man to mouse by intermediary hamster passage.[5] Rodent MM virus was found to be highly pathogenic for guinea pigs, producing symptoms and lesions similar to those induced by rodent SK virus.[6]

In the course of the early experiments with rodent SK virus certain observations were made which suggested the controlling effect of season upon the frequency with which transfer of the murine strain from mice to guinea pigs resulted in the evolution of paralysis. Thus, transfers of murine virus carried out during the winter months of 2 successive years caused a significantly higher incidence of paralysis in the injected guinea pigs than transfers carried out during the intervening summer months. The guinea pigs, however, which had escaped paralysis carried specific neutralizing substances in their serum and proved refractory to repeated reinoculation at later dates. The present communication reports additional observations on the same subject which serve to confirm and amplify these earlier findings.

The SK murine strain was propagated, in serial transmission, by intracerebral passage

[4] Toomey, J. A. and Takacs, W. S., *J. Bact.*, 1942, **43**, 87.

[5] Jungeblut, C. W. and Dalldorf, G., *Am. J. Publ. Health*, 1943, **33**, 169.

[6] Jungeblut, C. W., *J. Exp. Med.*, 1945, **81**, 275.

from mouse to mouse; similarly the SK cavian strain was propagated by serial intracerebral passage from guinea pig to guinea pig. At monthly intervals, intracerebral transfers were made of currently harvested murine virus from mouse to guinea pig. To further analyze the problem, mouse virus was harvested in the winter and, after cold storage in glycerine, was transferred intracerebrally to guinea pigs in the following summer; conversely, mouse virus harvested in the summer, after cold storage in glycerine, was transferred intracerebrally to guinea pigs in the following winter. The amount of virus (murine or cavian) used was 0.1 cc of a 10^{-1} viral brain suspension which represented from 10 to 20 minimal paralytic guinea pig doses; the guinea pigs weighed between 225 and 275 g and were of uniform stock, having been obtained from one dealer throughout this work. The number of animals involved, the dates of transfer, and the incidence of paralysis observed are recorded in Table I.

It is obvious from the data presented in Table I that serial propagation of fixed cavian virus in guinea pigs was not significantly influenced by seasonal fluctuation of any sort, the incidence of paralysis ranging between 75 and 100% through winter and summer periods alike. In contrast herewith, however, the mode of transmission of murine virus from mouse to guinea pig seemed to function more effectively in the winter than in the summer. Thus, current transfers of murine virus during the winter months of 2 years (Jan. '42-March '42; Dec. '42-March '43) paralyzed 69 or 77% respectively, of the injected guinea pigs, whereas current transfers during the summer months of the same 2 years (June '42-Sept. '42; June '43-Sept. '43) paralyzed only 41 or 40%, respectively, of the injected guinea pigs. While the percentages given for each year *per se* do not differ significantly, considered as a whole the data do assume statistical significance. It appears further that winter-harvested mouse virus, when tested in summer-guinea pigs, caused appreciably less paralysis (46 to 40%) than it had produced in winter-guinea pigs (69 to 77%). Again, in this case, we need the evidence of both years to assert statistical significance. It would finally seem that the incidence of paralysis obtained with summer-harvested mouse virus ran consistently higher in winter-guinea pigs (50 to 67%) than in summer-guinea pigs (41 to 40%), even though, in this instance, the number of animals involved is not sufficient to eliminate the element of chance. The possibility that the virus had undergone appreciable deterioration upon storage can be discounted since earlier experience had shown a remarkable stability of glycerinated virus when kept for 1 year in the icebox. We are indebted to Dr. John W. Fertig, professor of Biostatistics at the DeLamar Institute of Public Health, Columbia University Medical School, for the statistical appraisal of the figures given above.

The above data form a pattern, with a distinct trend, that has now been traced over a period of 3 continuous years. It seems reasonable enough to assume that the periodic drops in the incidence of paralysis observed in guinea pigs following injection with murine virus at different times of the year are caused, preponderantly, by seasonal variation in the receptivity of the host. However, since the disease in mice runs also a somewhat milder course during the summer months,[7] the possibility cannot be dismissed that concomitant changes in the quality of the virus at the source were a contributory factor. The observed phenomenon, in many ways, shows similarity with the well-known seasonal fluctuation in the response of guinea pigs to diphtherial toxin.[8] The precise mechanism of such seasonal variations in the susceptibility of laboratory animals to infectious or toxic agents is still obscure. However, to suspect that the experimental data—even though difficult to interpret at present—bear some valid relationship to the factors that control the epidemiology of the corresponding diseases in man, would be at least a justifiable working hypothesis.

Summary. Serial propagation of cavian poliomyelitis virus from guinea pig to guinea pig was not significantly influenced by seasonal fluctuation of any sort. However, the

[7] Jungeblut, C. W., Sanders, M. and Feiner, R. R., *J. Exp. Med.*, 1942, **75**, 611.

[8] Suedmersen, H. J. and Glenny, A. T., *J. Hyg.*, 1909, **9**, 399.

mode of transmission of murine poliomyelitis virus from mouse to guinea pig functioned more effectively in the winter than in the summer, as judged by the ratio between paralyzed and non-paralyzed animals. The described phenomenon appears to be due, preponderantly, to cyclic variations in the susceptibility of the guinea pig to the paralyzing effect of the virus when passed by intracerebral injection from one host to another.

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